BIOLOGY



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Biology

John W. Kimball

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It has always seemed to me that the many parts that make up the subject of biology are related to each other more like the nodes of a web than as a linear collection of independent topics. So I believe that the power of hypertext will be better suited to learning about biology than is the linear structure of a printed textbook. Another disadvantage of printed textbooks is the inevitable delay between the time that new advances in biology are reported and the time that they can become incorporated in a printed book (often several years). Material here can be updated promptly. So although some of this information has been drawn from the sixth edition of the author's text Biology published in 1994 by Wm. C. Brown, every effort has been made to adapt the material to the opportunities provided by an online text.

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UNIT 1: THE CHEMICAL BASIS OF LIFE

The following pages examine some of the principles of chemistry upon which an understanding of modern biology depends.

1.1: Mixtures and Compounds

1.2: Elements and Atoms

1.3: Electronegativity and types of Chemical Bonds

1.4: Noncovalent Bonding

1.5: Hydrogen Bonds

1.6: Acids and Bases

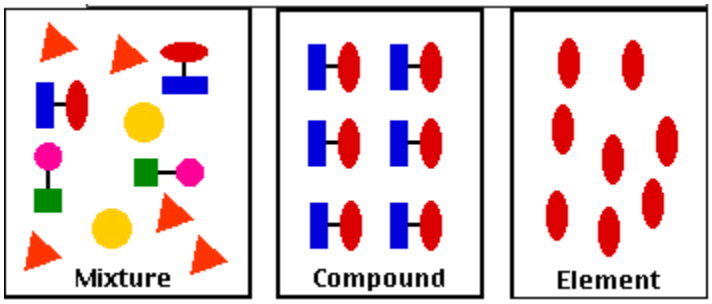
1.7: Molecular Weight and the Mole

1.8: pH

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1.1: Mixtures and Compounds

Mixtures are heterogeneous forms of matter. Mixtures are composed of **variable proportions** of molecules and atoms.



The composition of a mixture is variable with each components retaining its characteristic properties. Its components are easily separated. Examples of Mixtures: soil, ocean water and other **solutions, air,** the cytosol of a cell

n contrast, compounds are homogeneous forms of matter. Their constituent elements (atoms and/or ions) are always present in fixed proportions . Properties of compounds include

- The relative proportions of the elements in a compound are fixed.
- The components of a compound do **not** retain their individual properties. Both sodium and chlorine are poisonous; their compound, table salt (NaCl) is absolutely essential to life.
- It takes large inputs of energy to separate the components of a compound.

Examples of Compounds

- water (H_2O)
- table salt (NaCl)
- sucrose (table sugar, $C_{12}H_{22}O_{11}$)

Separating the Components of a Mixture

Most laboratory work in biology requires the use of techniques to separate the components of mixtures. This is done by exploiting some property that distinguishes the components, such as their relative

- size
- density
- solubility
- electrical charge

Dialysis

Dialysis is the separation of small solute molecules or ions (e.g., glucose, Na^+, Cl^-) from macromolecules (e.g., starch) by virtue of their differing rates of diffusion through a differentially permeable membrane.

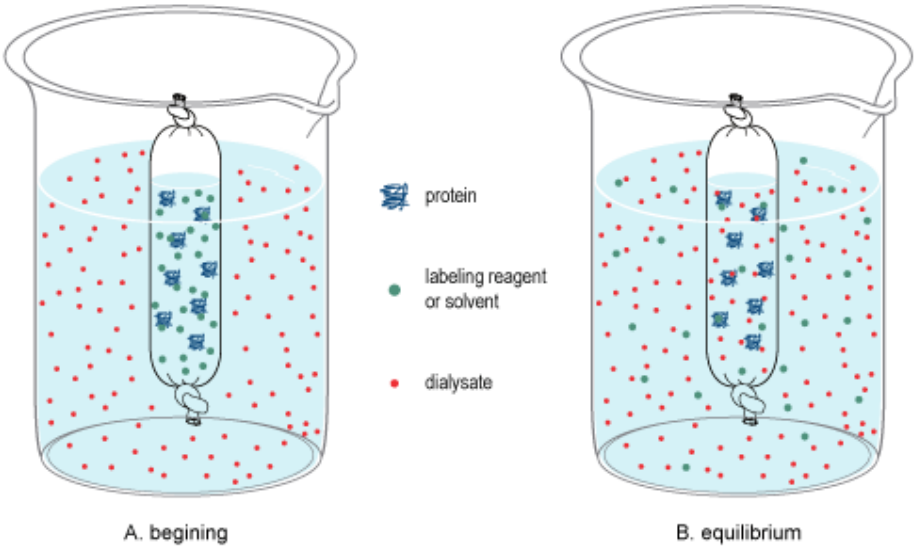


Figure 1.1.1: *Small-molecule dialysis using dialysis tubing. (CC-SA-BY-3.0; Potcherboy).*

As shown in Figure 1.1.1, the cellophane used to construct a bag is perforated with tiny pores that permit ions and small molecules to pass through, but exclude molecules with molecular weights greater than about 12,000. If a cellophane bag is mixed with a mixture of sugar and starch and place it in salt water, the sugar molecules (teal dots) will diffuse out into the water until **equilibrium** is reached; i.e., until their concentrations are equal on both sides of the membrane. Similarly, the salt (red dots) will diffuse into the bag. However, because of their large size, all the starch (big blue disks) will be retained within the tubing.

Chromatography

Chromatography is the term used for several techniques for separating the components of a mixture. The different types of chromatography techniques used are: paper chromatography, exclusion chromatography, and affinity chromatography.

Paper chromatography technique provides an easy way to separate the components of a mixture. A drop of mixture is placed in one corner of a square of absorbent paper.

- One edge of the paper is immersed in a solvent. **(a)**
- The solvent migrates up the sheet by capillary attraction.
- As it does so, the substances in the drop are carried along at different rates. **(b)**
- Each compound migrates at a rate that reflects
  - the size of its molecule and
  - its solubility in the solvent.
- After a second run at right angles to the first (often using a different solvent), the various substances will be spread out at distinct spots across the sheet, forming a **chromatogram**. **(c)**
- The identity of each spot can be determined by comparing its position with the position occupied by known substances under the same conditions.
- In many cases, a fragment of the paper can be cut away from the sheet and chemical analysis run on the tiny amount of substance in it.

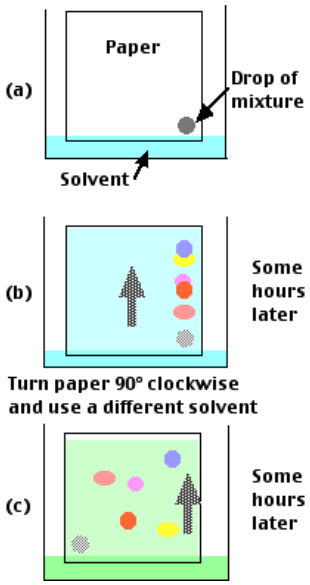
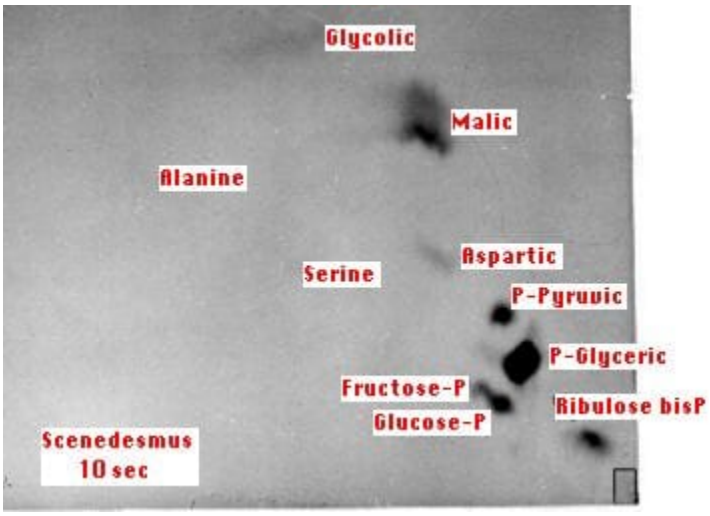
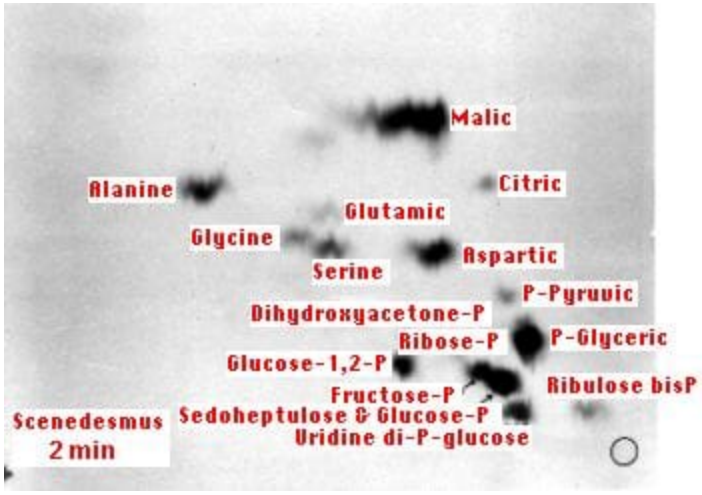


Figure 1.1.2: Paper chromatography

Autoradiography

If the mixture contains molecules that have been labeled with a radioactive isotope, these can be located by placing the chromatogram next to a sheet of X-ray film. The location of dark spots on the developed film (because of radiation emitted by the isotope) can be correlated with the position of the substances on the chromatogram.



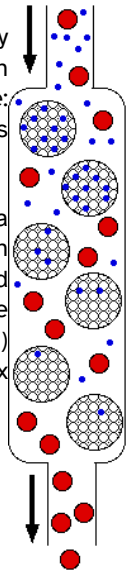


The above figures (courtesy of Dr. James A. Bassham) show autoradiograms of the type that were essential in working out the dark reactions of photosynthesis. The dark spots show the radioactive compounds produced after 10 secs (left) and 2 minutes (right) of photosynthesis by the green alga *Scenedesmus*. The alga was supplied with carbon dioxide labeled with ^{14}C, a radioactive isotope of carbon.

- At 10 seconds, most of the radioactivity is found in 3-phosphoglyceric acid ("P-Glyceric").
- At 2 minutes, phosphorylated 6-carbon sugars (glucose and fructose) have been synthesized as well as a number of amino acids.

The small rectangle and circle (lower right-hand corners) mark the spots where the cell extract was applied.

Exclusion chromatography

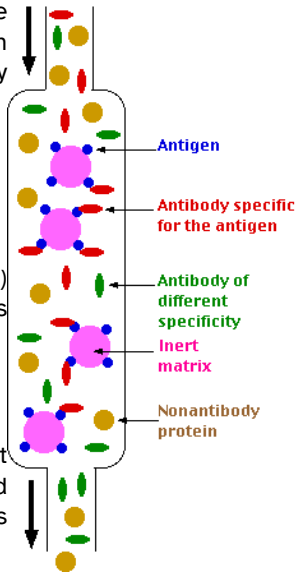


One of the most common problems in biochemical research is to separate the many components — usually macromolecules — in cell extracts and the like. Methods for separating the components of a mixture exploit such differences as size, electrical charge, and solubility in different solvents. of the molecules in it. One example: Electrophoresis which separates such macromolecules as proteins and DNA by their charge (and sometimes size as well).

Exclusion chromatography separates molecules on the basis of size. A column is filled with semi-solid beads of a polymeric gel that will admit ions and small molecules (blue) into their interior but not large ones (shown in red). When a mixture of molecules and ions dissolved in a solvent is applied to the top of the column, the smaller molecules (and ions) are distributed through a larger volume of solvent than is available to the large molecules. Consequently, the large molecules move more rapidly through the column, and in this way the mixture can be separated (fractionated) into its components. The porosity of the gel can be adjusted to exclude all molecules above a certain size. Sephadex and sepharose are trade names for gels that are available commercially in a broad range of porosities.

Affinity chromatography

The goal of affinity chromatography is to separate all the molecules of a particular specificity from the whole gamut of molecules in a mixture such as a blood serum. For example, the antibodies in a serum sample specific for a particular antigenic determinant can be isolated by the use of affinity chromatography.



The following steps are performed to achieve that:

Step 1

An **immunoadsorbent** is prepared. This consists of a solid matrix to which the antigen (shown in blue) has been coupled (usually covalently). Agarose, sephadex, derivatives of cellulose, or other polymers can be used as the matrix.

Step 2

The serum is passed over the immunoadsorbent. As long as the capacity of the column is not exceeded, those antibodies in the mixture specific for the antigen (shown in red) will bind (noncovalently) and be retained. Antibodies of other specificities (green) and other serum proteins (yellow) will pass through unimpeded.

Step 3

Elution. A reagent is passed into the column to release the antibodies from the immunoadsorbent. Buffers containing a high concentration of salts and/or low pH are often used to disrupt the noncovalent interactions between antibodies and antigen. A denaturing agent, such as 8 M urea, will also break the interaction by altering the configuration of the antigen-binding site of the antibody molecule.

Another, gentler, approach is to elute with a soluble form of the antigen. These compete with the immunoadsorbent for the antigen-binding sites of the antibodies and release the antibodies to the fluid phase.

Step 4

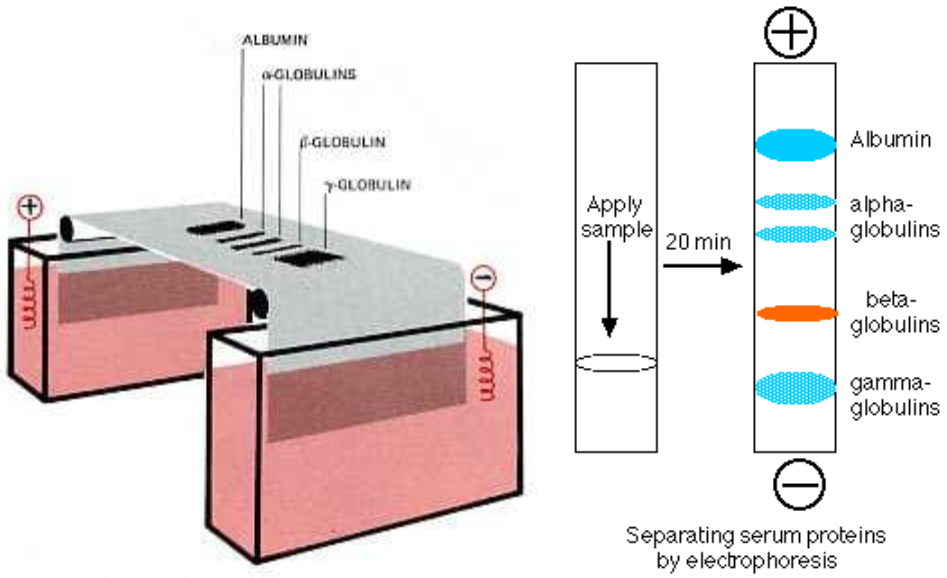
Dialysis. The eluate is then dialyzed against, for example, buffered saline in order to remove the reagent used for elution.

Electrophoresis

Electrophoresis uses a direct electric current to separate the components of a mixture by the differing **electrical charge**.

Example of Electrophoresis

Proteins in blood serum can be separated by electrophoresis.



- A drop of serum is applied in a band to a thin sheet of supporting material, like paper, that has been soaked in a slightly-alkaline salt solution.
- At pH 8.6, which is commonly used, all the proteins are negatively charged, but some more strongly than others.
- A direct current can flow through the paper because of the conductivity of the buffer with which it is moistened.
- As the current flows, the serum proteins move toward the positive electrode.
- The stronger the negative charge on a protein, the faster it migrates.
- After a time (typically 20 min), the current is turned off and the proteins stained to make them visible (most are otherwise colorless).
- The separated proteins appear as distinct bands.
- The most prominent of these and the one that moves closest to the positive electrode is **serum albumin**.
- The other proteins are the various serum globulins.

Pure Substances

Some of the pure substances isolated from mixtures cannot be further broken down. Oxygen (O_2) is an example. It is one of the **elements**; the fundamental building blocks of matter. Most pure substances are **compounds**. Table salt, sodium chloride (NaCl), is an example; water (H_2O) is another. If we pass an electrical current through molten NaCl, two new substances will be formed:

- **sodium**, a shiny metal so reactive that it must be stored out of contact with the air
- **chlorine**, a yellowish poisonous gas.

In this operation, a compound has been decomposed into its constitutive **elements**. Note the differences between separating the components of a mixture and those of a compound. The decomposition of NaCl required a large input of energy since the strong ionic bonds holding the Na and Cl atoms together must be broken. The ratio of the weights of the two products are always 23 parts of sodium to 35.5 parts of chlorine. This reflects the invariance of the ratio (1:1 in this case) of the number of atoms in a compound and the relative weights (23:35.5) of the atoms in table salt. The properties of the components of the compound are not the same as those of the compound itself. Both sodium and chlorine are hazardous to life; their compound, sodium chloride, is a vital ingredient of all animal diets.

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1.2: Elements and Atoms

Elements

Elements consist of only one kind of atom and cannot be decomposed into simpler substances. Our planet is made up of some **90 elements**. (Tiny amounts — sometimes only a few atoms — of additional elements have been made in nuclear physics laboratories, but they play no role in our story). Of these 90, only **25 or so** are used to build **living things**. The table shows the 11 most prevalent elements in the lithosphere (the earth's crust) and in the human body.

Living matter

- uses only a fraction of the elements available to it
- but, as the table shows, the relative proportions of those it does acquire from its surroundings are quite different from the proportions in the environment

So,

- the composition of living things is not simply a reflection of the elements available to them
  - For example, hydrogen, carbon, and nitrogen together represent less than 1% of the atoms found in the earth's crust but some 74% of the atoms in living matter.
- one of the properties of life is to take up certain elements that are scarce in the nonliving world and concentrate them within living cells.

  Some sea animals accumulate elements like vanadium and iodine within their cells to concentrations a thousand or more times as great as in the surrounding sea water. It has even been proposed that uranium be "mined" from the sea by extracting it from certain algae that can take up uranium from sea water and concentrate it within their cells.

Elemental composition of the lithosphere and the human body. Each number represents the percent of the total **number** of atoms present. For example, 47 of every 100 atoms found in a representative sample of the lithosphere are oxygen while there are only 19 atoms of carbon in every 10,000 atoms of lithosphere.

Composition of the Lithosphere		Composition of the Human Body	
Oxygen	47	Hydrogen	63
Silicon	28	Oxygen	25.5
Aluminum	7.9	Carbon	9.5
Iron	4.5	Nitrogen	1.4
Calcium	3.5	Calcium	0.31
Sodium	2.5	Phosphorus	0.22
Potassium	2.5	Chlorine	0.03
Magnesium	2.2	Potassium	0.06
Titanium	0.46	Sulfur	0.05
Hydrogen	0.22	Sodium	0.03
Carbon	0.19	Magnesium	0.01
All others	<0.1	All others	<0.01

There is still some uncertainty about the exact number of elements required by living things. Some elements, e.g., aluminum, are found in tiny amounts in living tissue, but whether they are playing an essential role or are simply an accidental acquisition (aluminum probably is) is sometimes difficult to determine.

Atoms

Each element is made up of one kind of atom. We can define an atom as the smallest part of an element that can enter into combination with other elements.

Structure of the atom

Each atom consists of a small, dense, **positively-charged** nucleus surrounded by much lighter, **negatively-charged electrons**. The nucleus of the simplest atom, the **hydrogen atom** (**H**), consists of a single **positively-charged proton**. Because of its single proton, the atom of hydrogen is assigned an **atomic number** of **1 and a single electron.** The charge of the electron is the same magnitude as that of the proton, so the atom as a whole is electrically neutral. Its proton accounts for almost all the weight of the atom.

The nucleus of the atom of the element **helium** (**He**) has **two protons** (hence helium has an **atomic number** of **2**) and **two neutrons**. Neutrons have the same weight as protons but no electrical charge. The helium atom has **two electrons** so that, once again, the atom as a whole is neutral.

The structure of each of the other kinds of atoms follows the same plan. From Lithium (At. No. = 3) to uranium (At. No. = 92), the atoms of each element can be listed in order of increasing atomic number. There are no gaps in the list. Each element has a unique atomic number and its atoms have one more proton and one more electron than the atoms of the element that precedes it in the list.

Electrons

Atomic Number	Element	Energy Levels or "shells"				
K	L	M	N	O		
1	Hydrogen (**H**)	1				
2	Helium (**He**)	2				
3	Lithium (**Li**)	2	1			
4	Beryllium (**Be**)	2	2			
5	Boron (**B**)	2	3			
6	Carbon (**C**)	2	4			
7	Nitrogen (**N**)	2	5			
8	Oxygen (**O**)	2	6			
9	Fluorine (**F**)	2	7			
10	Neon (**Ne**)	2	8			
11	Sodium (**Na**)	2	8	1		
12	Magnesium (**Mg**)	2	8	2		
13	Aluminum (**Al**)	2	8	3		
14	Silicon (**Si**)	2	8	4		
15	Phosphorus (**P**)	2	8	5		
16	Sulfur (**S**)	2	8	6		
17	Chlorine (**Cl**)	2	8	7		
18	Argon (**Ar**)	2	8	8		
19	Potassium (**K**)	2	8	8	1	
20	Calcium (**Ca**)	2	8	8	2	
21	Scandium (**Sc**)	2	8	9	2	
22	Titanium (**Ti**)	2	8	10	2	
23	Vanadium (**V**)	2	8	11	2	
24	Chromium (**Cr**)	2	8	13	1	
25	Manganese (**Mn**)	2	8	13	2	
26	Iron (**Fe**)	2	8	14	2	
27	Cobalt (**Co**)	2	8	15	2	
28	Nickel (**Ni**)	2	8	16	2	
29	Copper (**Cu**)	2	8	18	1	
30	Zinc (**Zn**)	2	8	18	2	
31	Gallium (**Ga**)	2	8	18	3	
32	Germanium (**Ge**)	2	8	18	4	
33	Arsenic (**As**)	2	8	18	5	
34	Selenium (**Se**)	2	8	18	6	

Atomic Number	Element	Energy Levels or "shells"				
35	Bromine (**Br**)	2	8	18	7	
36	Krypton (**Kr**)	2	8	18	8	
42	Molybdenum (**Mo**)	2	8	18	13	1
48	Cadmium (**Cd**)	2	8	18	18	2
50	Tin (**Sn**)	2	8	18	18	4
53	Iodine (**I**)	2	8	18	18	7

Electrons are confined to relatively discrete regions around the nucleus. The two electrons of helium, for example, are confined to a spherical zone surrounding the nucleus called the **K shell** or **K energy level**.

Lithium (At. No. = 3) has three electrons, two in the K shell and one located farther from the nucleus in the **L shell**. Being farther away from the opposite (+) charges of the nucleus, this third electron is held less tightly.

Each of the following elements, in order of increasing atomic number, adds one more electron to the L shell until we reach neon (At. No. = 10) which has eight electrons in the L shell.

Sodium places its eleventh electron in a still higher energy level, the **M shell**.

From sodium to **argon**, this shell is gradually filled with electrons until, once again, a maximum of eight is reached.

Note that after the K shell with its maximum of two electrons, the maximum number of electrons in any other **outermost** shell is **eight**.

As we shall see, the **chemical properties** of each element are strongly influenced by the **number of electrons in its outermost energy level** (shell).

This table shows the electronic structure of the atoms of elements 1 – 36 with those that have been demonstrated to be used by living things **shown in red**. Four elements of still higher atomic numbers that have been shown to be used by living things are also included.

The electronic structure of an atom plays the major role in its chemistry.

The pattern of electrons in an atom — especially those in the outermost shell — determines

- the **valence** of the atom; that is, the ratios with which it interacts with other atoms, and to a large degree,
- the **electronegativity** of the atom; that is, the strength with which it attracts other electrons.

Elements with the same number of electrons in their outermost shell show similar chemical properties.

Example 1: Fluorine, **chlorine**, **bromine**, and **iodine** each have 7 electrons in their outermost shell. These so-called **halogens** are also quite similar in their chemical behavior. When dissolved in water, for example, they all produce germicidal solutions.

Example 2: Those elements with 1, 2, or 3 electrons in their outermost shell are the **metals**.

Example 3: Those elements with 4, 5, 6, or 7 in their outermost shell are the **nonmetals**.

Example 4: Helium (with its 2), **neon**, **argon**, and **krypton** (each with 8) have "filled" their outermost shells. They are the so-called inert or "noble" gases. They have no chemistry at all. Under normal conditions they do not interact with other atoms. So, it is the number and arrangement of the electrons in the atoms of an element that establish the chemical behavior of that element.

This is how it works:

The atoms of an element interact with other atoms in such ways and ratios that they can "fill" their outermost shell with **8 electrons** (2 for hydrogen). They may do this by

- acquiring more electrons from another atom
- losing electrons to another atom
- sharing electrons with another atom

The number of electrons that an atom must acquire, or lose, or share to reach a stable configuration of 8 (2 for hydrogen) is called its **valence**.

Hydrogen, lithium, sodium, and potassium atoms all have a single electron in their outermost shell. Fluorine, chlorine, bromine, and iodine atoms all have 7. Any atom of the first group will interact with a single atom of any of the second group forming, HCl, NaCl, KI, etc. The result of all of these interactions is a pair of atoms each with an outermost shell like that of one of the inert gases: 2 for hydrogen, 8 for the others.

The elements with 2 electrons in their outermost shell interact with chlorine and the other halogens to form, e.g., $BeCl_2$, $MgCl_2$, $CaCl_2$. Again, the result is a pair of atoms each with a stable octet of electrons in its outermost shell.

The elements with 3 electrons in their outermost shell will interact with chlorine in a ratio of 1:3, forming BCl_3, $AlCl_3$.

Carbon atoms, with their 4 electrons in the L shell interact with chlorine to form CCl_4.

Nitrogen, with its 5 outermost electrons, interacts with hydrogen atoms in a ratio of 1:3, forming ammonia (NH_3).

Oxygen and **sulfur**, with their 6 outermost electrons react with hydrogen to form water (H_2O) and hydrogen sulfide (H_2S).

What determines whether a pair of atoms swap or share electrons?

The answer is their **relative electronegativities**. If two atoms differ greatly in their affinity for electrons; that is, in their electronegativity, then the strongly electronegative atom will take the electron away from the weakly electronegative one.

Example: Na (weakly electronegative) gives up its single electron to an atom of chlorine (strongly electronegative) to form NaCl. The sodium atom now has only 10 electrons but still 11 protons so there is a net positive charge of one on the atom. Similarly, chlorine now has one more electron than proton so its now has a net negative charge of 1. Electrically charged atoms are called **ions**. The mutual attraction of opposite electrical charges holds the ions together by **ionic bonds**.

Example: Carbon and hydrogen are both only weakly electronegative so neither can remove electrons from the other. Instead they achieve a stable configuration by sharing their outermost electrons forming **covalent bonds** of CH_4.

Isotopes

The number of protons in the nucleus of its atoms, which is its **atomic number**, defines each element. However, the nuclei of a given element may have varying numbers of neutrons. Because neutrons have weight (about the same as that of protons), such atoms differ in the **atomic weight**.

Atoms of the same element that differ in their atomic weight are called **isotopes**.

Atomic weights are expressed in terms of a standard atom: the isotope of carbon that has 6 protons and 6 neutrons in its nucleus. This atom is designated **carbon-12** or $^{12}\mathbf{C}$. It is arbitrarily assigned an atomic weight of 12 **daltons** (named after John Dalton, the pioneer in the study of atomic weights). Thus a dalton is 1/12 the weight of an atom of ^{12}C. Both protons and neutrons have weights very close to 1 dalton each. Carbon-12 is the most common isotope of carbon. Carbon-13 (^{13}C) with 6 protons and 7 neutrons, and carbon-14 (^{14}C) with 6 protons and 8 neutrons are found in much smaller quantities.

Isotopes as "tracers"

One can prepare, for example, a carbon compound used by living things that has many of its normal ^{12}C atoms replaced by ^{14}C atoms. Carbon-14 happens to be radioactive. By tracing the fate of radioactivity within the organism, one can learn the normal

pathway of this carbon compound in that organism. Thus ^{14}C serves as an isotopic "label" or "tracer".

The basis of this technique is that the weight of the nucleus of an atom has little or no effect on the chemical properties of that atom. The chemistry of an element and the atoms of which it is made — whatever their atomic weight — is a function of the **atomic number** of that element. As long as the atom had 6 protons, it is an atom of carbon irrespective of the number of neutrons. Thus while 6 protons and 8 neutrons produce an isotope of carbon, ^{14}C, 7 protons and 7 neutrons produce a totally-different element, nitrogen-14.

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1.3: Electronegativity and types of Chemical Bonds

Electronegativity

The electronegativity of an atom is a measure of its affinity for electrons. The atoms of the various elements differ in their affinity for electrons.

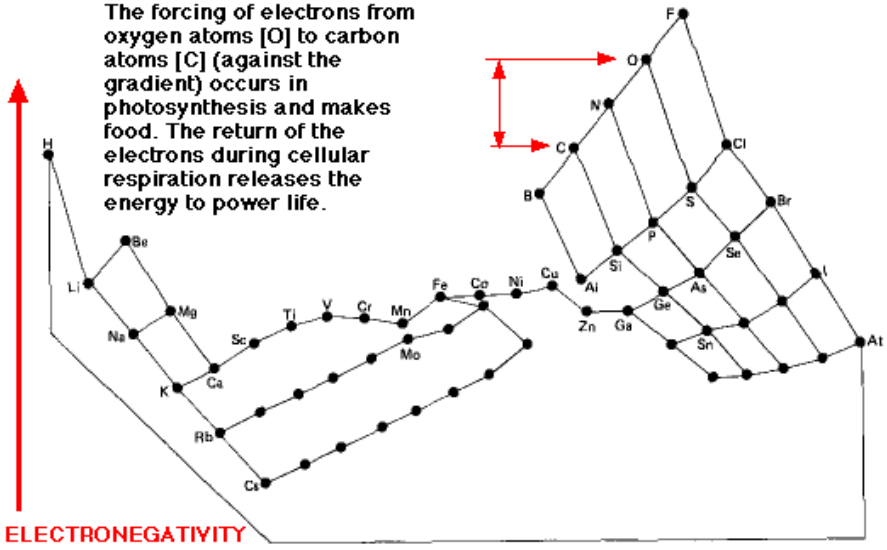


Figure 1.3.1

This image distorts the conventional periodic table of the elements so that the greater the electronegativity of an atom, the higher its position in the table. Although fluorine (F) is the most electronegative element, it is the electronegativity of runner-up oxygen (O) that is exploited by life. The shuttling of electrons between carbon (C) and oxygen (O) atoms powers life.

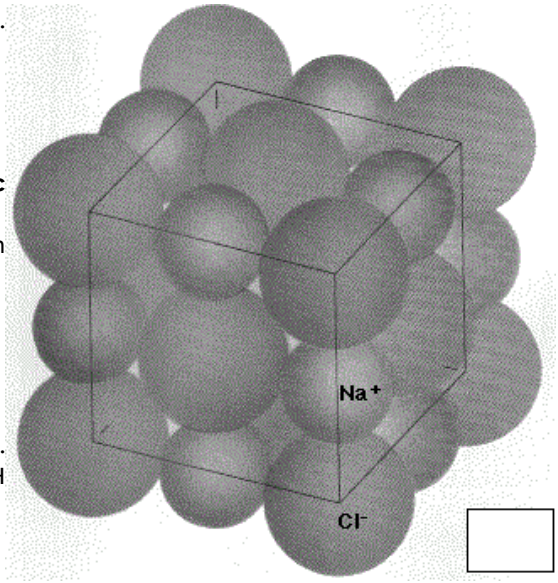
1. Moving electrons against the gradient (O to C) — as occurs in photosynthesis — requires energy (and stores it).
2. Moving electrons down the gradient (C to O) — as occurs in cellular respiration — releases energy.

The relative electronegativity of two interacting atoms also plays a major part in determining what kind of chemical bond forms between them.

Chemical Bonds

Three main types of chemical bonds:Ionic Bond, Covalent Bond, Polar Covalent Bond.

Ionic Bond



Example of an ionic bond is : Sodium (Na) and Chlorine (Cl) = Ionic Bond. There is a large difference in electronegativity between Na and Cl atoms, so

- the chlorine atom takes an electron from the sodium atom
- converting the atoms into **ions** (Na^{+}) and (Cl^{-})
- These are held together by their opposite electrical charge forming **ionic bonds**
- Each sodium ion is held by 6 chloride ions while each chloride ion is, in turn, held by 6 sodium ions
- Result: a **crystal lattice** (not molecules) of common table salt (NaCl)

Covalent Bond

Example of a covalent bond is: Carbon (C) and Hydrogen (H) = Covalent Bond. There is only a small difference in electronegativity between the C and H atoms, so

- the two atoms share the electrons
- Result: a **covalent bond** (depicted as C**:**H or C**-**H)
- The atoms are held together by their mutual affinity for their shared electrons
- An array of atoms held together by covalent bonds forms a true **molecule**

Polar Covalent Bond

Example of a polar covalent bond is: Hydrogen (H) and Oxygen (O) = Polar Covalent Bond. There is a moderate difference in electronegativity, causing the oxygen atom to pull the electron of the hydrogen atom closer to itself. This results in a **polar covalent bond. Oxygen does this with 2 hydrogen atoms to form a molecule of water**

Molecules, like water, with polar covalent bonds are themselves polar; that is, have partial electrical charges across the molecule and may be attracted to each other (as occurs with water molecules). These species are good solvents for polar and/or hydrophilic compounds may form hydrogen bonds.

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1.4: Noncovalent Bonding

Noncovalent Bonding

Noncovalent bonding does not involve sharing of electrons. Instead it:

- holds the two strands of the DNA double helix together (hydrogen bonds)
- folds polypeptides into such secondary structures as the alpha helix and the beta conformation
- enables **enzymes** to bind to their **substrate**
- enables **antibodies** to bind to their **antigen**
- enables transcription factors to bind to each other
- enables transcription factors to bind to DNA
- enables proteins (e.g. some hormones) to bind to their receptor
- permits the assembly of such macromolecular machinery as
  - ribosomes
  - actin filaments
  - microtubules
- and many more

There are three principle kinds of noncovalent forces:

- ionic interactions
- hydrophobic interactions
- hydrogen bonds

Ionic Interactions

At any given pH, proteins have charged groups that may participate in binding them to each other or to other types of molecules. For example, as the figure shows, negatively-charged carboxyl groups on aspartic acid (Asp) and glutamic acid (Glu) residues may be attracted by the positively-charged free amino groups on lysine (Lys) and arginine (Arg) residues.

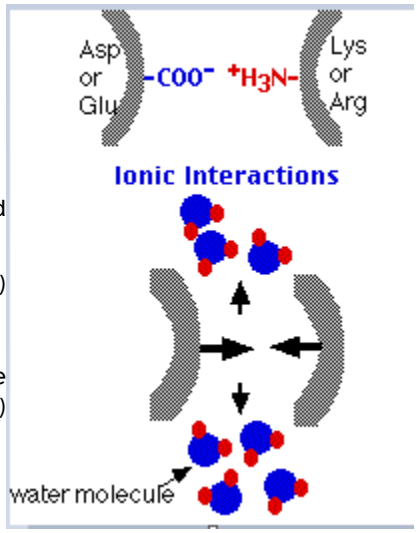
Ionic interactions are highly sensitive to

- **changes in pH**.

  As the pH **drops**,

  - H^+ bind to the carboxyl groups (COO^-) of aspartic acid (Asp) and glutamic acid (Glu), neutralizing their negative charge, and
  - H^+ bind to the unoccupied pair of electrons on the N atom of the amino (NH_2) groups of lysine (Lys) and arginine (Arg) giving them a positive charge

  The result: Not only does the net charge on the molecule change (it becomes more positive) but many of the opportunities that its R groups have for ionic (electrostatic) interactions with other molecules and ions are altered.



As the pH **rises**,

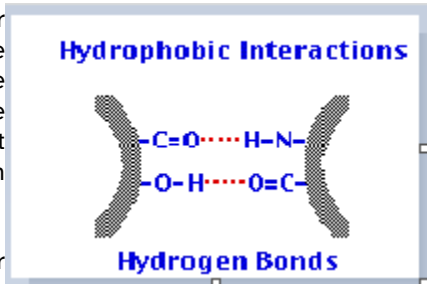
- H^+ are removed from the COOH groups of Asp and Glu, giving them a negative charge (COO^-), and
- H^+ are removed from the NH_3^+ groups of Lys and Arg removing their positive charge

The result: Again the net charge on the molecule changes (it becomes more negative) and, again, many of the opportunities its R groups have for electrostatic interactions with other molecules or ions are altered.

- **salt concentration**

Increasing salt concentration reduces the strength of ionic binding by providing competing ions for the charged residues.

Hydrophobic Interactions



The side chains (R groups) of such amino acids as phenylalanine and leucine are nonpolar and hence interact poorly with polar molecules like water. For this reason, most of the nonpolar residues in globular proteins are directed toward the interior of the molecule whereas such polar groups as aspartic acid and lysine are on the surface exposed to the solvent. When nonpolar residues are exposed at the surface of two different molecules, it is energetically more favorable for their two "oily" nonpolar surfaces to approach each other closely displacing the polar water molecules from between them.

The strength of hydrophobic interactions is not appreciably affected by changes in pH or in salt concentration.

Hydrogen Bonds

Hydrogen bonds can form whenever

- a strongly electronegative atom (e.g., oxygen, nitrogen) approaches
- a hydrogen atom which is **covalently** attached to a second strongly-electronegative atom

Some common examples:

- between the –C=O group and the H-N– group of nearby peptide bonds in proteins giving rise to the alpha helix and beta configuration

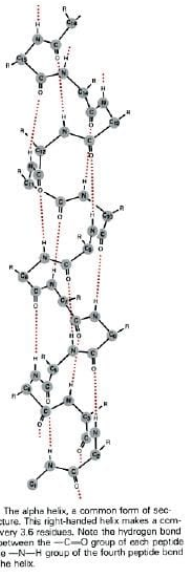


Figure 4.21 The alpha helix, a common form of secondary structure. This right-handed helix makes a complete turn every 3.6 residues. Note the hydrogen bond that forms between the —C=O group of each peptide bond and the —N—H group of the fourth peptide bond below it in the helix.

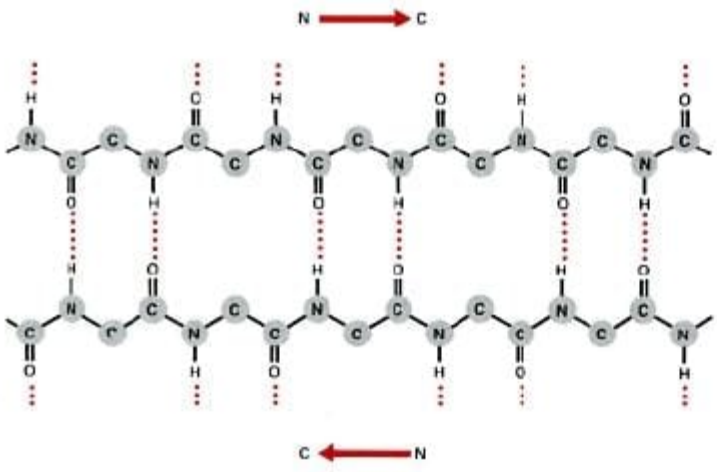
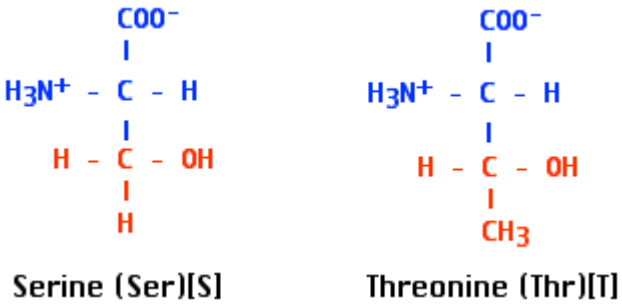


Figure 4.22 The beta-pleated sheet, another common example of secondary structure. The chains are held parallel to each other by the hydrogen bonds that form between them.

- Between –C=O groups and hydroxyl (H-O–) groups in



  - serine and threonine residues of proteins and
  - sugars

Noncovalent interactions are individually weak but collectively strong.

All three forms of noncovalent interactions are individually weak (on the order of 5 kcal/mole) as compared with a covalent bond (with its 90–100 kcal/mole of bond energy). And what strength these interactions do have requires that the interacting groups can approach each other closely (an angstrom or less). So we can conclude that all the examples given at the top of the page require:

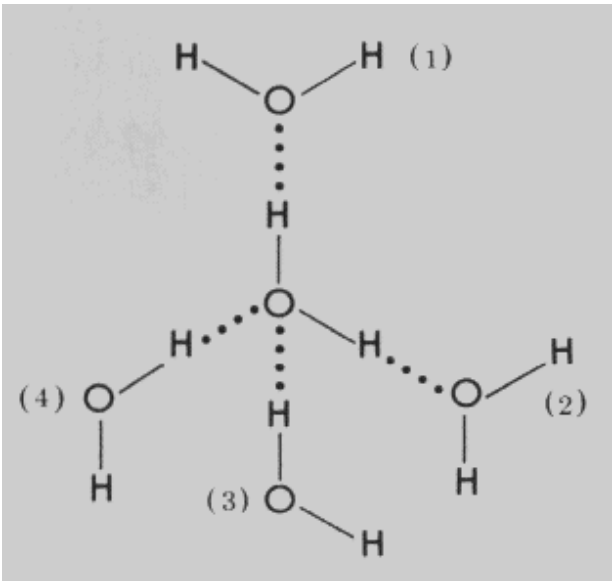
- a substantial number of noncovalent interactions working together to hold the structures together
- a surface topography that enables substantial areas of two interacting surfaces to approach each other closely; that is, they must fit each other

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1.5: Hydrogen Bonds

Hydrogen Bonds

Polar molecules, such as water molecules, have a weak, partial negative charge at one region of the molecule (the oxygen atom in water) and a partial positive charge elsewhere (the hydrogen atoms in water). Thus when water molecules are close together, their positive and negative regions are attracted to the oppositely-charged regions of nearby molecules. The force of attraction, shown here as a dotted line, is called a **hydrogen bond**. Each water molecule is hydrogen bonded to four others.



The hydrogen bonds that form between water molecules account for some of the essential — and unique — properties of water.

- The attraction created by hydrogen bonds keeps water liquid over a wider range of temperature than is found for any other molecule its size.
- The energy required to break multiple hydrogen bonds causes water to have a high heat of vaporization; that is, a large amount of energy is needed to convert liquid water, where the molecules are attracted through their hydrogen bonds, to water vapor, where they are not.

Two outcomes of this:

- The evaporation of sweat, used by many mammals to cool themselves, cools by the large amount of heat needed to break the hydrogen bonds between water molecules.
- Reduction of temperature extremes near large bodies of water like the ocean.

The hydrogen bond has only 5% or so of the strength of a covalent bond. However, when many hydrogen bonds can form between two molecules (or parts of the same molecule), the resulting union can be sufficiently strong as to be quite stable.

Multiple hydrogen bonds

- hold the two strands of the DNA double helix together
- hold polypeptides together in such secondary structures as the alpha helix and the beta conformation
- help enzymes bind to their substrate
- help antibodies bind to their antigen
- help transcription factors bind to each other
- help transcription factors bind to DNA

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1.6: Acids and Bases

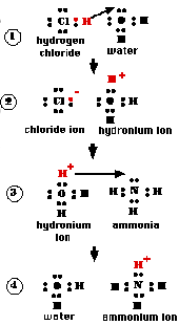
Acids

Acids are substances that donate protons (hydrogen ions, H^+) to bases.

Bases

Bases are substances that accept protons from acids.

Example of Acid and Base formation

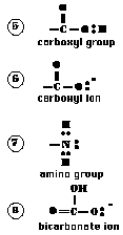


Hydrogen chloride (HCl) is a gas. Its two atoms are held together by a shared pair of electrons. However, the chlorine atom is so much more **electronegative** than hydrogen, that the bond between them is **polar covalent**.

When hydrogen chloride is bubbled through water, the nucleus of the hydrogen atom leaves and takes up residence at one of the unshared pairs of electrons in the water molecule. However, its electron remains behind still attached to the chlorine atom. **"1"** This ionization produces:

- a **chloride ion** (Cl^-)
- a **hydronium ion** ($H3O^+$). **"2"**

The resulting mixture is called **hydrochloric acid**.



Now let us bubble **ammonia** gas (NH3) through the hydrochloric acid. Ammonia molecules have one pair of unshared electrons and these have a greater affinity for a proton than do the unshared electrons in the water molecule. Consequently, the proton shifts again (**"3"**) to form a new ion, the **ammonium ion** ($NH4^+$) and water (**"4"**).

Because both the HCl molecule and the hydronium ion are proton donors, they meet the definition of an **acid**.

The water molecule in the first example and the ammonia in the second example accept protons; therefore each is a **base**.

While HCl is found in living systems (e.g., the gastric juice secreted by the stomach), the most common acids in biology are those containing the **carboxyl group** (**"5"**).

The proton of the carboxyl group is easily removed forming the **carboxyl ion** (**"6"**).

Acetic acid (CH3COOH) is a common example of a carboxylic acid. When mixed with water, some of the protons on its -COOH group are attracted to the unshared electron pairs of water molecules. Hydronium ions ($H3O^+$) and acetate ions ($CH3COO^-$) result. Vinegar is a dilute solution of acetic acid.

Ammonia is also found (in low concentrations) in living matter. But the most common bases are those molecules that contain an **amino group** (**"7"**). The unshared pair of electrons serves as a proton acceptor, as it does in the ammonia molecule.

Bicarbonate ions (**"8"**) also serve as an important base in living tissue.

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1.7: Molecular Weight and the Mole

Molecular Weight

The weight of a molecule is the sum of the weights of the atoms of which it is made. The unit of weight is the **dalton**, one-twelfth the weight of an atom of ^{12}C. Thus the **molecular weight** (**MW**) of water is 18 daltons. (We shall ignore the tiny error introduced by the presence of traces of other isotopes - ^{17}O, ^{18}O, and ^{2}H among the predominant ^{1}H and ^{16}O atoms.)

Why is it important to know the molecular weight of a compound?

For an example, let us assume that you want to study the response of honeybees to solutions of various kinds of sugars. One way to do this would be to make up several different solutions and see which one the bees prefer to harvest.

You might offer the bees the choice between, say, a 35% solution of sucrose (common table sugar) and a 35% solution of glucose (a natural component of honey). This would involve, in each case, dissolving 350 parts by weight (e.g., grams) of sugar in 650 parts (g) of water, thus producing 1000 g of each solution. But there is a problem with this approach. The willingness of the honeybee to respond to the presence of sugar dissolved in water is dependent on the **number** of sugar molecules in a given volume of the solution.

The sucrose molecule (MW = 342) is almost twice as heavy as the glucose molecule (MW = 180). So a 35% solution of glucose would contain almost twice as many molecules as a 35% solution of sucrose. To correct the problem, you should make the solution with the weights of sucrose and glucose in a ratio of 342:180. Then you would have the same concentration of molecules in each; that is, drop for drop, each solution would contain the same number of molecules.

Mole

A mole is the quantity of a substance whose weight in grams is equal to the molecular weight of the substance. If you weight out exactly 342 grams (g) of sucrose, you will have weighed out **1 mole** of it. Thus 1 mole of glucose weighs 180 g. Furthermore, if you dissolve 1 mole of a substance in enough water to make 1 **liter** (**L**) of solution, you have made a **1-molar** (**1 *M***) solution.

A **1 *M*** solution of these sugars would probably be too strong for the experiment with the bees. It might be better to make up a liter of each solution containing 34.2 g and 18.0 g respectively. Such solutions would be designated **one-tenth molar** (**0.1 *M***) solutions. Drop for drop, these two solutions would still contain exactly the same number of molecules because they are of the same **molarity**.

Avogadro's Number

How many molecules are there in a mole?

Solution

The number is approximately 6×10^{23}. This number is called Avogadro's number after the chemist who first attempted to determine it.

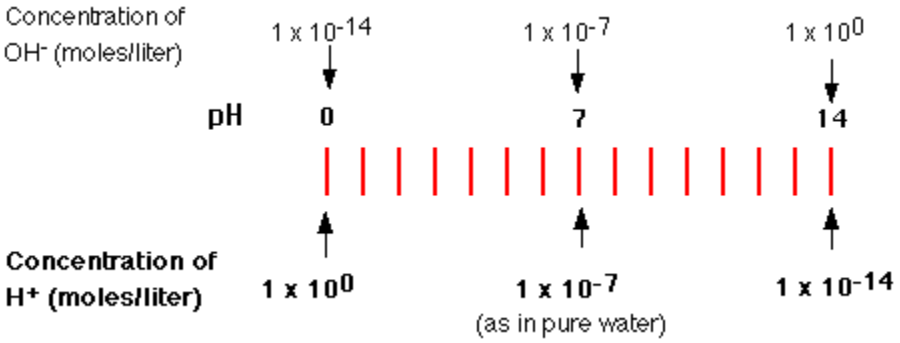
Avogadro's number applies to a mole of any substance: molecule or ion. Thus we can properly refer to a mole of hydrogen ions (1 g).

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1.8: pH

pH is a measure of the concentration of **hydrogens ions** (= $\mathbf{H^+}$) (= **protons**) in a solution. Numerically it is the **negative** logarithm of that concentration expressed in moles per liter (**M**).

Pure water spontaneously dissociates into ions, forming a 10^{-7} M solution of H^+ (and OH^-). The negative of this logarithm is 7, so the pH of pure water is 7.



Solutions with a higher concentration of H^+ than occurs in pure water have pH values below 7 and are **acidic**. Solutions containing molecules or ions that reduce the concentration of H^+ below that of pure water have pH values above 7 and are **basic** or **alkaline**.

Is pH important? Yes!

The properties of most proteins, enzymes for example, are sensitive to pH.

As the pH drops,

- H^+ bind to the carboxyl groups (COO^-) of aspartic acid (Asp) and glutamic acid (Glu), neutralizing their negative charge, and
- H^+ bind to the unoccupied pair of electrons on the N atom of the amino (NH_2) groups of lysine (Lys) and arginine (Arg) giving them a positive charge.

The result: Not only does the net charge on the molecule change (it becomes more positive) but many of the opportunities that its R groups have for ionic interactions with other molecules and ions are altered.

As the pH rises,

- H^+ are removed from the COOH groups of Asp and Glu, giving them a negative charge (COO^-), and
- H^+ are removed from the NH_3^+ groups of Lys and Arg removing their positive charge.

The result: Again the net charge on the molecule changes (it becomes more negative) and, again, many of the opportunities its R groups have for ionic interactions with other molecules or ions are altered.

The pH of the **cytosol** within a human cell is about 7.4. BUT, this value masks the pH differences that are found in various compartments within the cell. For example,

- The interior of **lysosomes** is much more acidic (as low as pH 4) than the cytosol, and the enzymes within work best at these low pH values.
- The pH differential created within **chloroplasts** by the energy of the sun is harnessed to synthesize ATP which, in turn, powers the synthesis of food.
- The pH differential created within **mitochondria** during the respiration of food is harnessed to the synthesis of ATP which, in

turn, powers most of the energy-consuming activities of the cell such as locomotion and biosynthesis of cell components.

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UNIT 2: THE MOLECULES OF LIFE

2.1: Organic Molecules

2.2: Hydrocarbons

2.3: Fats

2.4: Phospholipids

2.5: Cholesterol

2.6: Carbohydrates

2.7: Amino Acids

2.8: Enantiomers

2.9: Polypeptides

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2.11: Rules of Protein Structure

2.12: Glycoproteins

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2.14: Proteomics

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- Thumbnail: Cellulose molecular structure (CC BY-SA 3.0 Unported; Pintor4257 via Wikipedia)

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2.1: Organic Molecules

Functional groups

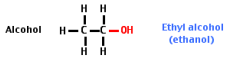
The various functional groups include:

- hydroxyl group (-OH)
- carboxyl group [-COOH]
- carbonyl group (-C=O)
- amino group -NH_2

Organic Molecules

Various organic molecules formed by these groups are as follows:

Alcohols

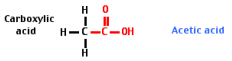


Organic molecules with a **hydroxyl group** (-OH).

Methanol [CH_3OH] and ethanol (beverage alcohol)[CH_3CH_2OH] are common examples.

Sugars are also alcohols.

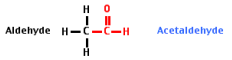
Carboxylic Acids

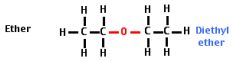


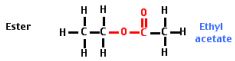
Contain one or more **carboxyl groups** [-COOH].

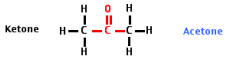
Many of the intermediates in the breakdown of foodstuffs by cellular respiration are carboxylic acids.

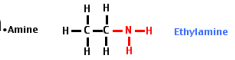
Aldehydes

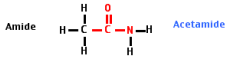












Contain a carbon atom to which is attached one hydrogen atom and — by a double bond — one oxygen atom.

Formaldehyde [HCHO] is a powerful disinfectant and preservative (it denatures proteins).

Acetaldehyde is produced during the conversion of pyruvic acid to ethanol when yeast ferment sugars. The converse is also true — acetaldehyde is produced in the liver as it metabolizes ingested ethanol (and may be the prime culprit in a "hangover").

Phosphoglyceraldehyde is an intermediate in glycolysis and the "dark reaction" of photosynthesis

Ethers

Formed when two carbon atoms are linked by an oxygen atom.

Diethyl ether is a commonly-used anesthetic.

Esters

The removal of a molecule of water between the -OH group of an alcohol and the -OH group of a

- carboxylic acid (-COOH) [shown in the diagram] or

- phosphoric acid

produces an ester.

- Fats are triesters of three fatty acids and glycerol (the alcohol).
- Phospholipids are also esters.
- **Nucleotides** are esters of nucleosides and phosphoric acid.
- The nucleotides of DNA and RNA are linked by a double ester linkage called a **phosphodiester** bond.

Ketones

Organic molecules with a **carbonyl group** (-C=O) between two hydrocarbon portions.

Ketones are synthesized in the liver, usually from fatty acids.

When glucose metabolism is suppressed, during starvation or in diabetics, fatty acids are used as a source of energy. But instead of entering the citric acid cycle, the **acetyl-CoA** produced from them is converted into the ketone acetoacetate. Some of this is then converted into **acetone** (which can be smelled on the breath of patients whose diabetes is out of control).

Amines

Organic molecules with an **amino group**, -NH_2. Some examples:

- all the amino acids (lysine has two of them).
- the thyroid hormones thyroxine (T_4) and triiodothyronine (T_3)
- Many neurotransmitters:
  - adrenaline and noradrenaline
  - dopamine
  - serotonin (5-hydroxytryptamine)
  - histamine

Amides

Amides are organic molecules containing a carbonyl group (-C=O) attached to a nitrogen atom. The **peptide bond** between the amino acids linked in a polypeptide is also called an **amide bond**.

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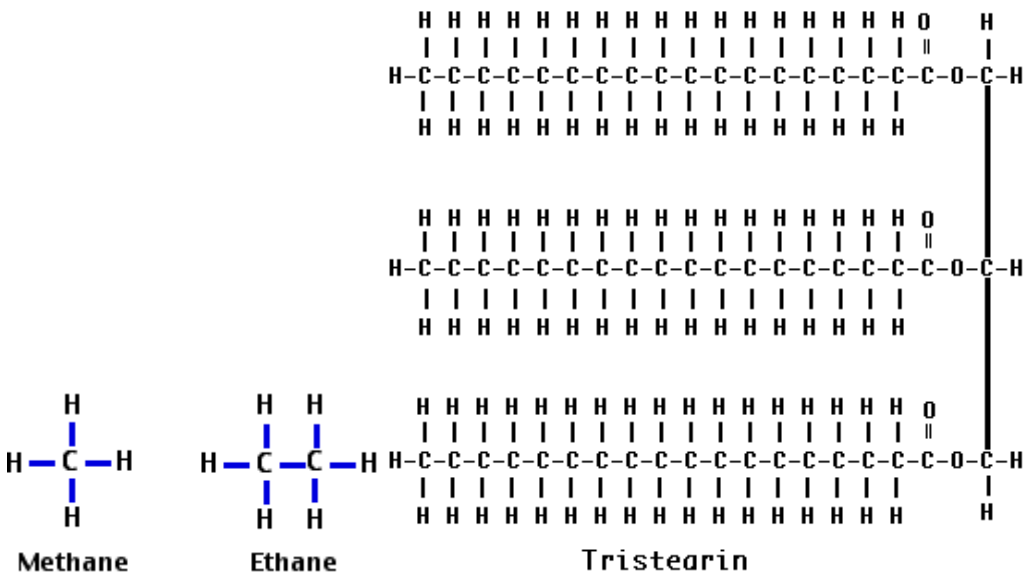
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2.2: Hydrocarbons

Hydrocarbons are organic molecules that consist exclusively, or primarily, of carbon and hydrogen atoms. They come in two flavors: (1) aliphatic hydrocarbons that consist of linear chains of carbon atoms and (2) aromatic hydrocarbons that which consist of closed rings of carbon atoms.

Aliphatic Hydrocarbons



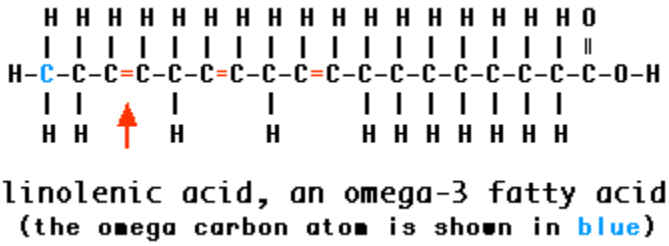
The simplest is methane, CH_4. Next is ethane, C_2H_6.

The fatty acids in fats are aliphatic hydrocarbons. If a chain holds all the hydrogen atoms it can, the molecule is said to be **saturated**. The fatty acids in **tristearin** are all saturated.

If two adjacent carbon atoms each lose a hydrogen atom, a double bond forms between them. Such a molecule is said to be **unsaturated**.

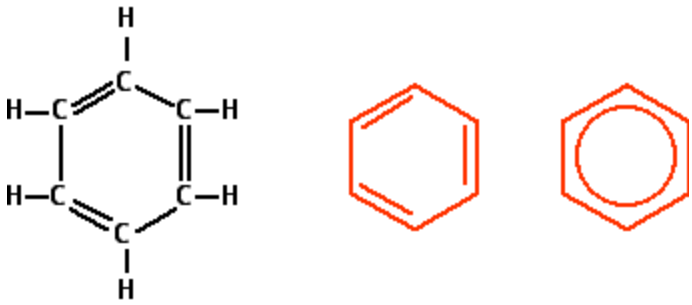
Example : Ethylene $H_2C{=}CH_2$

The fatty acids in trilinolein and linolenic acid are examples of unsaturated fatty acids



Aromatic Hydrocarbons

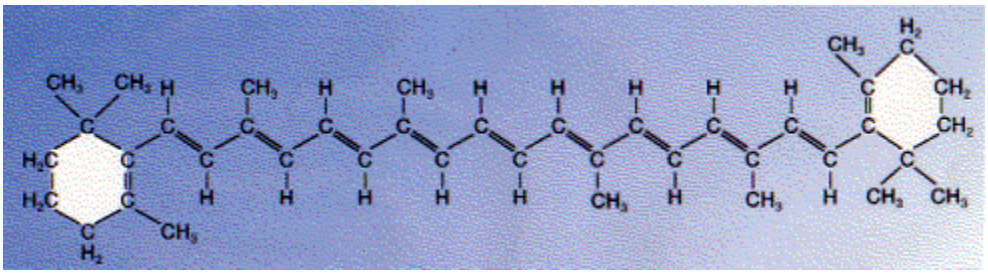
The building block of aromatic hydrocarbons is the benzene ring. The arrangement of atoms is shown on the left. The version in the center is often used to simplify diagrams of molecular structures. The three double bonds are not restricted to the positions shown but are free to pass around the ring. This is sometimes indicated by drawing the benzene ring as it is on the far right.



Benzene depicted in three ways

Some examples of biological molecules that incorporate the benzene ring:

- the amino acids tyrosine and phenylalanine
- cholesterol and its various derivatives, such as the sex hormones: estrogens and testosterone
- the herbicide, 2,4-D



The carotenoid, **beta-carotene**, is a hydrocarbon that has both aliphatic and aromatic portions.

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2.3: Fats

Fat molecules are made up of four parts: a molecule of **glycerol** (on the right) and three molecules of **fatty acids.** Each fatty acid consists of a hydrocarbon chain with a carboxyl group at one end. The glycerol molecule has three hydroxyl groups, each able to interact with the carboxyl group of a fatty acid. Removal of a water molecule at each of the three positions forms a **triglyceride**. The three fatty acids in a single fat molecule may be all alike (as shown here for **tristearin**) or they may be different. They may contain as few as 4 carbon atoms or as many as 24.

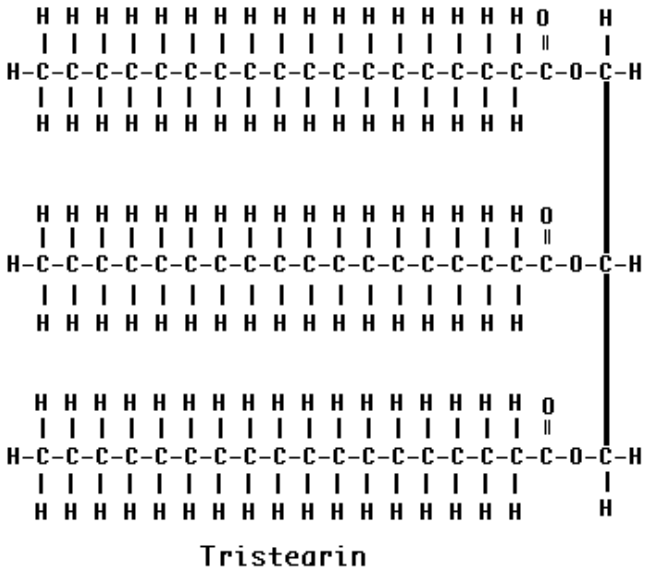


Figure 3.0.1: tristearin

Because fatty acids are synthesized from fragments containing two carbon atoms, the number of carbon atoms in the chain is almost always an even number. In animal fats, 16-carbon (palmitic acid) and 18-carbon (stearic acid - shown here) fatty acids are the most common.

Unsaturated Fats

Some fatty acids have one or more double bonds between their carbon atoms. They are called unsaturated because they could hold more hydrogen atoms than they do. **Monounsaturated** fats have a single double bond in their fatty acids and **polyunsaturated** fats, such as **trilinolein** shown here, have two or more.

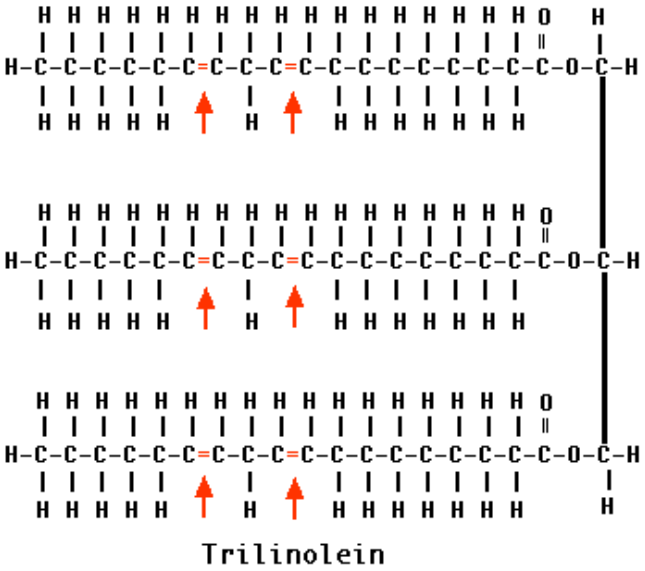


Figure 3.0.2: trilinolein

Double bonds are rigid and those in natural fats introduce a kink in the molecule. This prevents the fatty acids from packing close together and as a result, unsaturated fats have a lower melting point than do saturated fats. Because most of them are liquid at room temperature, we call them oils. Corn oil, canola oil, cottonseed oil, peanut oil, and olive oil are common examples. As this list suggests, plant fats tend to be unsaturated (therefore "oils"). Fats from such animals as cattle tend to be saturated.

Trans Fatty Acids

The most abundant (and least expensive) source of fat is from plant oils but many cooking applications, particularly baked products, need solid fats. The food industry uses **hydrogenated** oils for things like **shortening** and **margarine.** In hydrogenation, plant oils are exposed to hydrogen at a high temperature and in the presence of a catalyst.

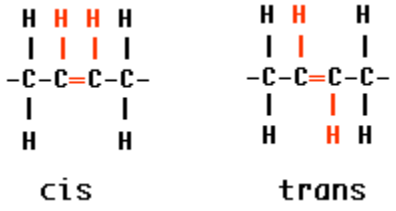


Figure 3.0.3: Trans vs. Cis fatty acid

Two things result:(1) some double bonds are converted into single bonds and (2) other double bonds are converted from **cis** to **trans** configuration. Both these effects straighten out the molecules so they can lie closer together and become solid rather than liquid.

Omega fatty acids

One system for naming unsaturated fatty acids is to indicate the position of the **first** double bond counting from the opposite end from the carboxyl group. That terminal carbon atom (shown here in blue) is called the **omega** carbon atom. Thus a monounsaturated fatty acid with its single double bond after carbon #3 (counting from and including the omega carbon) is called an omega-3 fatty acid. But so is a **polyunsaturated** fatty acid, such as linolenic acid (shown here), if its first double bond is in that position.

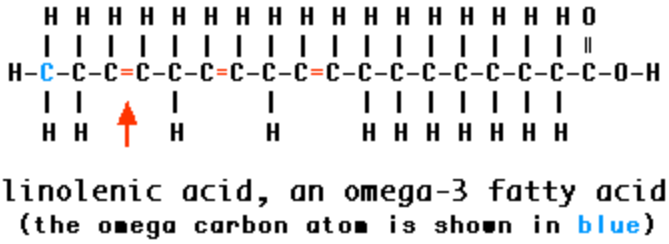


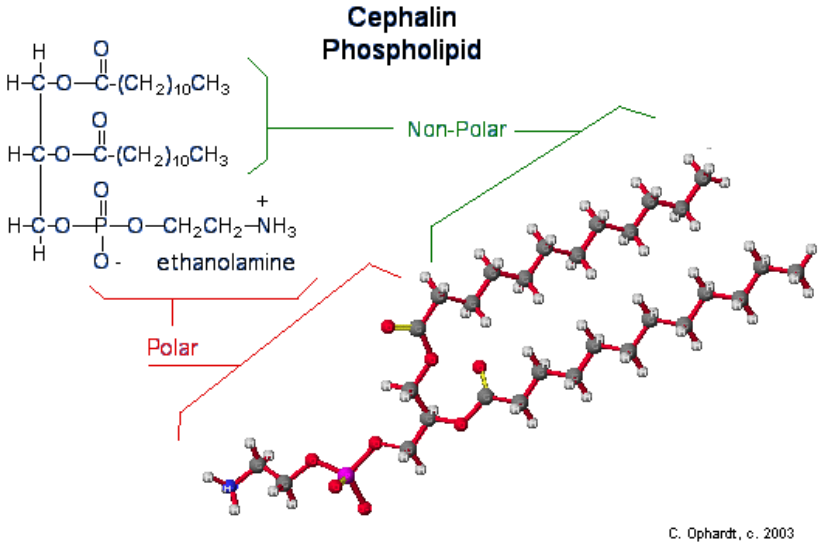
Figure 3.0.4: Linolenic Acid

Some studies have suggested that omega-3 fatty acids help protect against cardiovascular disease. For this reason, a Dietary Reference Intake (DRI) of 1.1 grams/day for women (1.6 for men) was established in September 2002.

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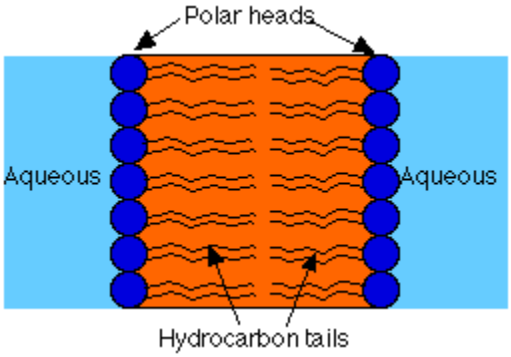
2.4: Phospholipids

Phospholipids are fat derivatives in which one fatty acid has been replaced by a phosphate group and one of several nitrogen-containing molecules.



***Example 2.4.1**: Phosphatidyl ethanolamine (also known as cephalin)*

The hydrocarbon chains are **hydrophobic** (as in all fats). However, the charges on the phosphate and amino groups (in red) make that portion of the molecule **hydrophilic**. The result is an **amphiphilic** molecule.

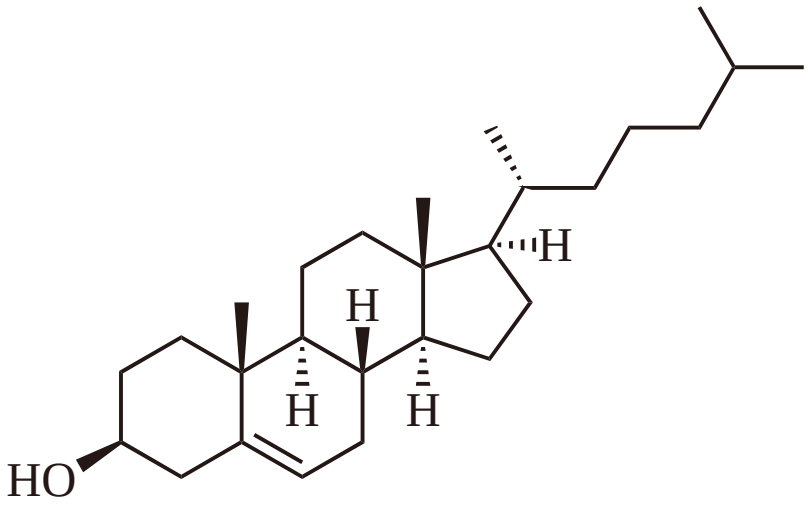


Phospholipids like phosphatidyl ethanolamine are major constituents of **cell membranes**. These molecules form a **phospholipid bilayer** with their hydrophilic (polar) heads facing their aqueous surroundings (e.g., the cytosol) and their hydrophobic tails facing each other.

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2.5: Cholesterol

The cholesterol molecule is a **steroid** that is essential to life. It has also been responsible for 17 Nobel Prizes, countless pages of reports in scientific journals and the popular press, and mounting anxiety on the part of health-conscious people. The human body contains about 100 g of cholesterol. Most of this is incorporated in the membranes from which cells are constructed and is an indispensable component of them. The insulating layers of *myelin* wound around neurons are especially rich in cholesterol.



The structure of Cholesterol

Uses of Cholesterol

Cholesterol is starting ingredient for the synthesis of the steroid hormones including progesterone, estrogens, androgens (e.g., testosterone), glucocorticoids (e.g., cortisol), and mineralocorticoids (e.g., aldosterone). Cholesterol is also the precursor from which the body synthesizes vitamin D.

Another major use of cholesterol is the synthesis of **bile acids**. These are synthesized in the liver from cholesterol and are secreted in the bile. They are essential for the absorption of fat from the contents of the intestine. A clue to the importance of cholesterol is that more than 90% of the bile acids are not lost in the feces but are reabsorbed from the lower intestine and recycled to the liver. There is some loss, however, and to compensate for this and to meet other needs, the liver synthesizes some 1500–2000 mg of new cholesterol each day. It synthesizes cholesterol from the products of fat metabolism.

There is also an unceasing transport of cholesterol in the blood between the liver and all the other tissues. Most of this cholesterol travels as **low density lipoproteins** (**LDL**s). Each LDL particle is a sphere filled with ~1,500 molecules of cholesterol complexed with fatty acids and coated with a layer of phospholipids and a single molecule of a protein called apolipoprotein B (apoB). Cells that need cholesterol trap and ingest LDLs by **receptor-mediated endocytosis**.

Problems caused by cholesterol

Cholesterol can also create problems.

- High levels of LDL cholesterol lead to the development of **atherosclerosis**: cholesterol-rich deposits (plaques) that form on the inside of blood vessels and predispose to heart attacks.
- Cholesterol in the bile can crystallize to form gall stones that may block the bile ducts.

Typical lipid values in humans

The level of cholesterol in the blood is measured in milligrams per deciliter (**mg/dl**), which is equivalent to parts per 100,000. The levels range from less than 50 in infants to an average of 215 in adults and to 1,200 or more in individuals suffering from a rare, inherited disorder called **familial hypercholesterolemia**. For those of us in the normal range, approximately two-thirds of our cholesterol is transported as LDLs. Most of the rest is carried by so-called **high density lipoproteins** (**HDL**s).

Because of their relationship to cardiovascular disease, the analysis of serum lipids has become an important health measure. The table shows the range of typical values as well as the values above (or below) which the subject may be at increased risk of developing atherosclerosis.

LIPID	Typical values (mg/dl)	Desirable (mg/dl)
Cholesterol (total)	170–210	<200
LDL cholesterol	60–140	<130
HDL cholesterol	35–85	>40
Triglycerides	40–150	<135

- Total cholesterol is the sum of
  - HDL cholesterol
  - LDL cholesterol and
  - 20% of the triglyceride value
- Note that
  - **high LDL** values are **bad**, but
  - **high HDL** values are **good** (because HDL cholesterol transports cholesterol from the tissues back to the liver where it is secreted in the bile).
- Using the various values, one can calculate a
  cardiac risk ratio = **total cholesterol** divided by **HDL cholesterol**
- A cardiac risk ratio greater than 7 is considered a warning.

In May of 2001, a panel of the National Institutes of Health recommended a more aggressive attack on reducing cholesterol levels in the U.S. population. In addition to a better diet and more exercise, they urged that many more people at risk of developing heart disease, such as

- smokers
- diabetics
- people with high blood pressure and/or
- obesity

be put on cholesterol-lowering drugs.

There are several types:

- drugs that interfere with the ability of the liver to synthesize cholesterol by blocking the action of the enzyme HMG-CoA reductase. These are the "statins", e.g., **lovastatin** (Mevacor®), **pravastatin** (Pravachol®), **atorvastatin** (Lipitor®).
- insoluble powders ("colestipol", "cholestyramine") that bind to bile acids in the intestine so that instead of being reabsorbed they are eliminated in the feces. In compensation, the liver increases its consumption of blood-borne cholesterol. The main drawback to these drugs is that they are gritty powders and must be consumed in rather large amounts.
- nicotinic acid (niacin);
- "fibric acids" such as gemfibrozil and clofibrate.

Careful attention to diet may by itself lead to a reduction in cholesterol levels. In one study, men with high (>265 mg/dl) levels were able to lower these an average of 3.5% (10 mg/dl) by diet alone. Their diets were low in fat as well as low in cholesterol, and it was not — and still is not — clear as to what aspect of the diet contributed to the modest reduction. Cholesterol is made from fat and lowering the proportion of fat in the diet will probably help. Favoring unsaturated fats over saturated fats appears to be beneficial. There is little evidence that lowering one's intake of cholesterol is, by itself, useful. An average intake of cholesterol of 300–500 mg per day is joined in the intestine by several times that amount that has been synthesized by the liver and appears

to have little or no effect on blood levels of cholesterol. So when choosing between the pat of butter and the pat of margarine, it is not the 30-odd mg of cholesterol in the butter (vs. 0 in the margarine) but its high content of saturated fat (over 3 times that in the margarine) that is probably significant.

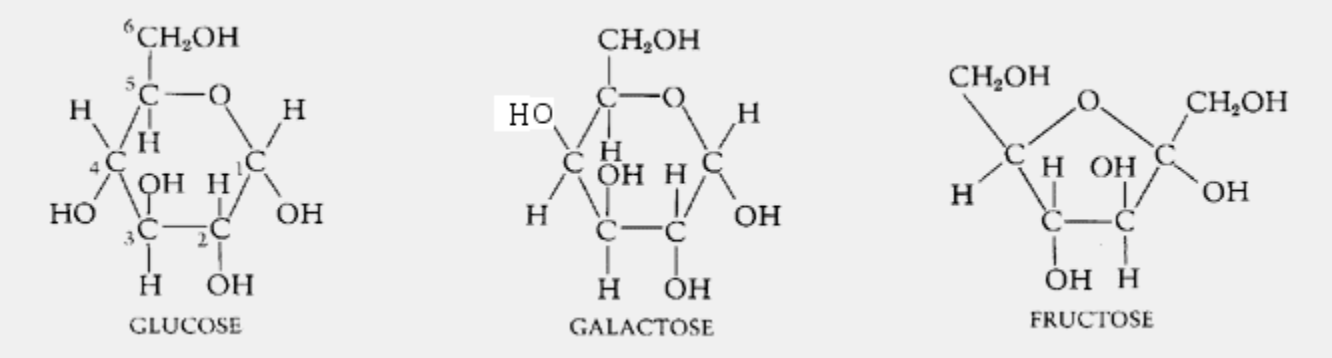
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2.6: Carbohydrates

Carbohydrates have the general molecular formula CH_2O, and thus were once thought to represent "hydrated carbon". However, the arrangement of atoms in carbohydrates has little to do with water molecules. Starch and cellulose are two common carbohydrates. Both are macromolecules with molecular weights in the hundreds of thousands. Both are polymers (hence "**polysaccharides**"); that is, each is built from repeating units, monomers, much as a chain is built from its links. The monomers of both starch and cellulose are the same: units of the sugar **glucose**.

Monosaccharides

Three common sugars share the same molecular formula: $C_6H_{12}O_6$. Because of their six carbon atoms, each is a **hexose**.



They are:

- glucose, "blood sugar", the immediate source of energy for cellular respiration
- **galactose**, a sugar in milk (and yogurt)
- **fructose**, a sugar found in honey

Although all three share the same molecular formula ($C_6H_{12}O_6$), the arrangement of atoms differs in each case. Substances such as these three, which have identical molecular formulas but different structural formulas, are known as **structural isomers**. Glucose, galactose, and fructose are "single" sugars or **monosaccharides**. Two monosaccharides can be linked together to form a "double" sugar or **disaccharide**.

Disaccharides

Three common disaccharides:

- sucrose — common table sugar = glucose + fructose
- **lactose** — major sugar in milk = glucose + galactose
- **maltose** — product of starch digestion = glucose + glucose

Although the process of linking the two monomers is rather complex, the end result in each case is the loss of a hydrogen atom (H) from one of the monosaccharides and a hydroxyl group (OH) from the other. The resulting linkage between the sugars is called a **glycosidic bond**. The molecular formula of each of these disaccharides is

$$C_{12}H_{22}O_{11} = 2C_6H_{12}O_6 - H_2O \tag{2.6.1}$$

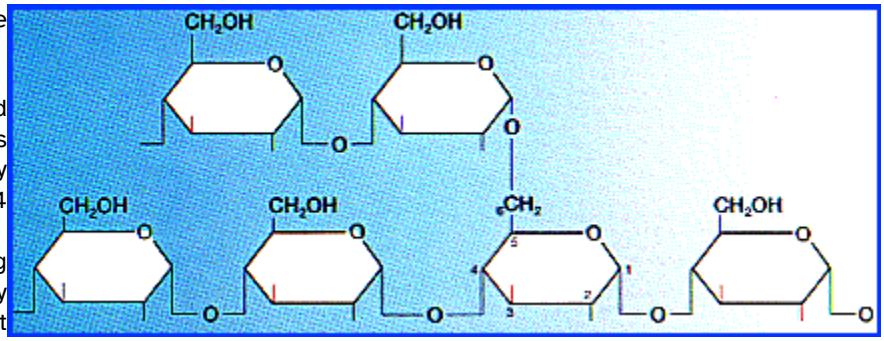
All sugars are very soluble in water because of their many hydroxyl groups. Although not as concentrated a fuel as fats, sugars are the most important source of energy for many cells. Carbohydrates provide the bulk of the calories (4 kcal/gram) in most diets, and starches provide the bulk of that. Starches are polysaccharides.

Polysaccharides

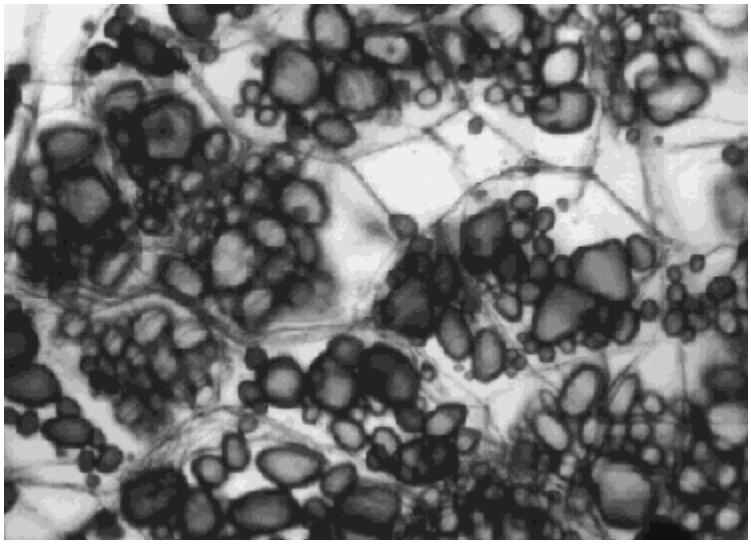
There are three primary polysaccharide polymer systems of interest: Starches, Glycogen and Cellulose.

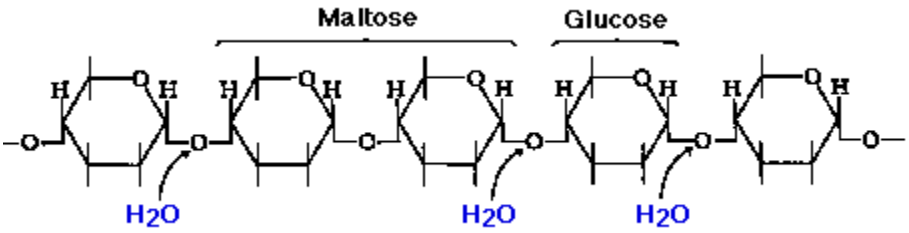
Starches

Starches are polymers of glucose. Two types are found:



- amylose consists of linear, unbranched chains of several hundred glucose residues (units). The glucose residues are linked by a glycosidic bond between their #1 and #4 carbon atoms.
- **amylopectin** differs from amylose in being highly branched. At approximately every thirtieth residue along the chain, a short side chain is attached by a glycosidic bond to the #6 carbon atom (the carbon above the ring). The total number of glucose residues in a molecule of amylopectin is several thousand.





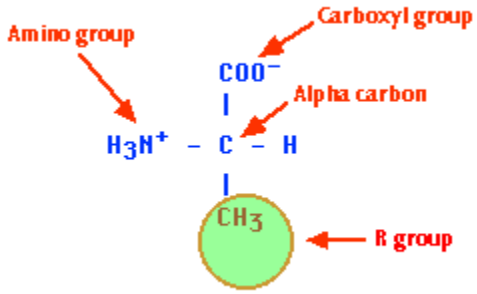
Potato starch Amylase

Figure 2.6.3: This electron micrograph (courtesy of R. D. Preston) shows the cellulose fibrils in the cell wall of a green alga. These long, rigid fibrils are a clear reflection of the nature of the cellulose molecules of which they are composed.

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2.7: Amino Acids

Amino acids are the building blocks (monomers) of proteins. 20 different amino acids are used to synthesize proteins. The **shape** and other properties of each protein is dictated by the precise sequence of amino acids in it.



Each amino acid consists of an **alpha carbon atom** to which is attached

- a hydrogen atom
- an amino group (hence "amino" acid)
- a carboxyl group (-COOH). This gives up a proton and is thus an acid (hence amino "acid")
- one of 20 different "R" groups. It is the structure of the R group that determines which of the 20 it is and its special properties. The amino acid shown here is **Alanine**

Table 2.7.1: Types of Amino Acids. For each amino acid both three-letter and single letter codes are given

Alanine	Ala	A	hydrophobic
Arginine	**Arg**	**R**	free amino group makes it basic and hydrophilic
Asparagine	**Asn**	**N**	carbohydrate can be covalently linked ("N-linked) to its -NH
Aspartic acid	**Asp**	**D**	free carboxyl group makes it acidic and hydrophilic
Cysteine	**Cys**	**C**	oxidation of their sulfhydryl (-SH) groups link 2 Cys (S-S)
Glutamic acid	**Glu**	**E**	free carboxyl group makes it acidic and hydrophilic
Glutamine	**Gln**	**Q**	moderately hydrophilic
Glycine	**Gly**	**G**	so small it is amphiphilic (can exist in any surroundings)
Histidine	**His**	**H**	basic and hydrophilic
Isoleucine	**Ile**	**I**	hydrophobic
Leucine	**Leu**	**L**	hydrophobic
Lysine	**Lys**	**K**	strongly basic and hydrophilic
Methionine	**Met**	**M**	hydrophobic
Phenylalanine	**Phe**	**F**	very hydrophobic
Proline	**Pro**	**P**	causes kinks in the chain
Serine	**Ser**	**S**	carbohydrate can be covalently linked ("O-linked") to its -OH
Threonine	**Thr**	**T**	carbohydrate can be covalently linked ("O-linked") to its -OH
Tryptophan	**Trp**	**W**	scarce in most plant proteins

Alanine	Ala	A	hydrophobic
Tyrosine	**Tyr**	**Y**	a phosphate or sulfate group can be covalently attached to its -OH
Valine	**Val**	**V**	hydrophobic

The Essential Amino Acids

Humans must include adequate amounts of 9 amino acids in their diet.

- Histidine
- Isoleucine
- Leucine
- Lysine
- Methionine (and/or cysteine)
- Phenylalanine (and/or tyrosine)
- Threonine
- Tryptophan
- Valine

These "essential" amino acids cannot be synthesized from other precursors. However, cysteine can partially meet the need for methionine (they both contain sulfur), and tyrosine can partially substitute for phenylalanine. Two of the essential amino acids, **lysine** and **tryptophan**, are poorly represented in most plant proteins. Thus strict vegetarians should ensure that their diet contains sufficient amounts of these two amino acids. 19 of the 20 amino acids listed above can exist in two forms in three dimensions.

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2.8: Enantiomers

In three-dimensional (3D) space, the four covalent bonds of carbon atoms point toward the corners of a regular tetrahedron. The molecule represented to the below is methane (CH_4).

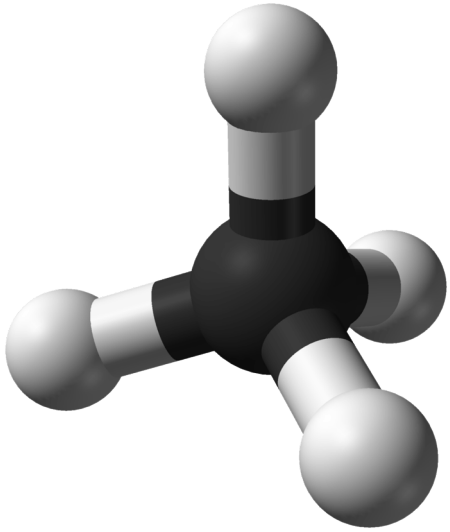


Figure 2.8.1: 3-D representation of methane. Image used wtih permission (public domain; credit Ben Mills)

Whenever a carbon atom has four different structures bonded to it, two different molecules can be formed.

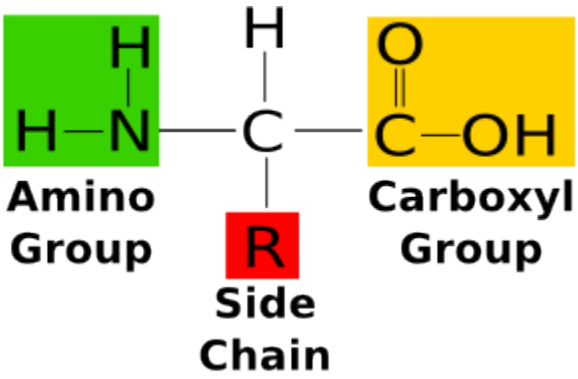
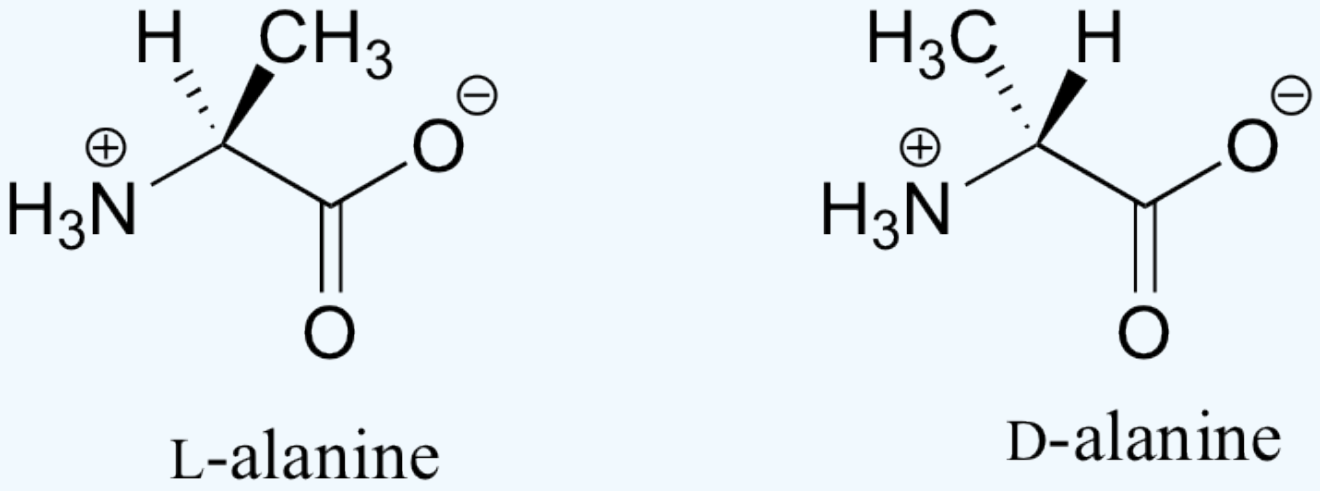


Figure 2.8.2: Basic Amino Acid Structure. Photo Credit: Credit: Yassine Mrabet

Example 2.8.1: Alanine

The amino acid **alanine**.



Bonded to its alpha carbon atom are four different groups:

- a **carboxyl** group (COO^-)
- an **amino** group ($NH3^+$)
- a **methyl** group ($CH3$)(its **R group**)
- a **hydrogen** atom

If you orient the molecule so that you look along it **from** the COO^- group **to** the $NH3^+$ group, the methyl (R) group can extend out to the left, forming **L-alanine** (shown on the left) or to the right, forming **D-alanine** (on the right). Although they share the same chemical formula, they are not interchangeable any more than a left-hand glove is interchangeable with right-hand glove.

19 of the 20 amino acids used to synthesize proteins can exist as **L**- or **D**- enantiomorphs. The exception is glycine, which has two (indistinguishable) hydrogen atoms attached to its alpha carbon. **L** amino acids are used exclusively for protein synthesis by all life on our planet. (Some **D** amino acids are used for other purposes).

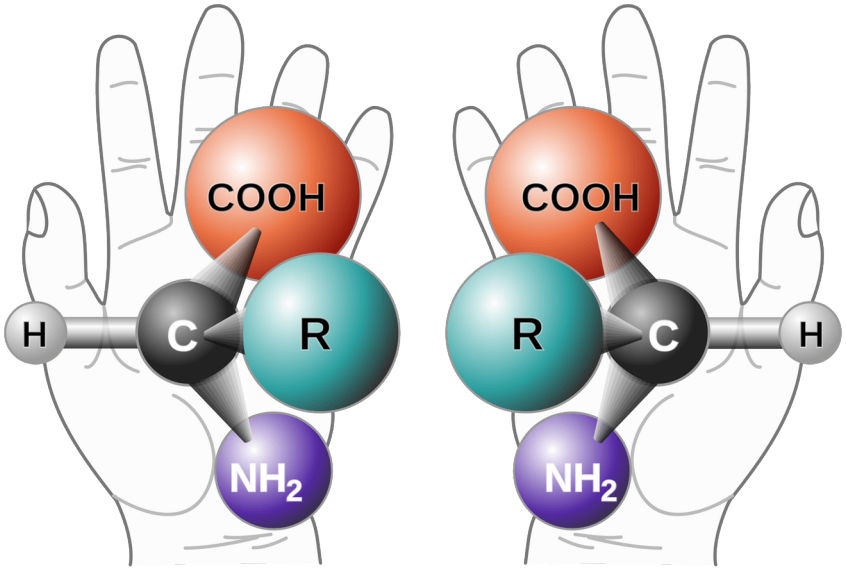


Figure 2.8.3: Two enantiomers of a generic ***chiral*** *amino acid.*

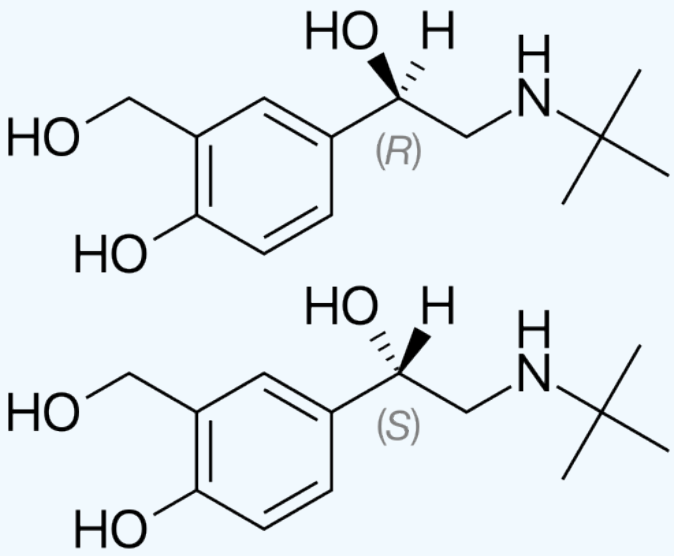
Does chirality really matter? **Yes**. The function of a protein is determined by its shape. A protein with a D amino acid instead of L

will have its R group sticking out in the wrong direction (Figure 2.8.3).

Many other kinds of organic molecules exist as enantiomers. Usually only one form is active in biological systems. For example, if one form binds to a receptor protein on the surface of a cell, the other probably cannot. With their protein catalysts (enzymes), cells usually synthesize only one form. However, chemical synthesis in the laboratory or pharmaceutical factory usually produces equal amounts of the two enantiomers — called a racemic mixture.

Example 2.8.2: Albuterol

The drug **albuterol** (e.g., Proventil®) contains equal amounts of two enantiomers. Only one of them is effective, and the other may be responsible for the occasional unpleasant side-effects associated with the drug (which is used to dilate the bronchi, e.g, during an attack of asthma). The active form can now be synthesized pure, and — called **levalbuterol** (Xopenex®) — is available by prescription.



Two enantiomers of albuterol: (R)-(−)-salbutamol (top) and (S)-(+)-salbutamol (bottom)

Enantiomers are also called optical isomers because their solutions rotate the plane of polarized light passing through them. If one enantiomer rotates light in the clockwise direction, a solution of the other enantiomer will rotate it in the opposite direction.

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2.9: Polypeptides

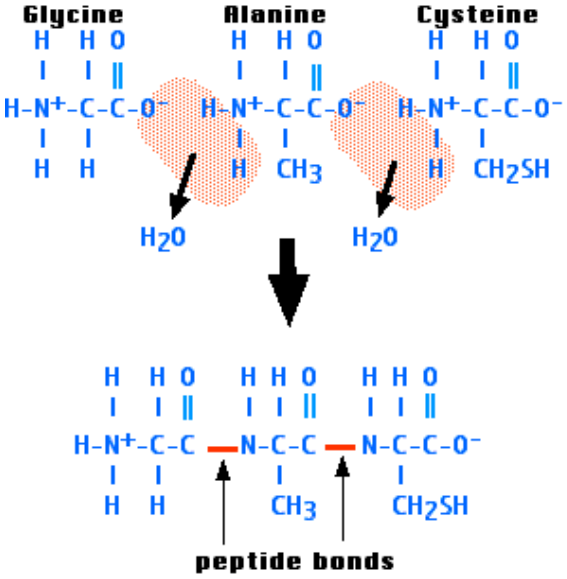


Figure 2.9.2: A longer peptide.

The sequence of amino acids in a polypeptide is dictated by the codons in the messenger RNA (mRNA) molecules from which the polypeptide was translated. The sequence of codons in the mRNA was, in turn, dictated by the sequence of codons in the DNA from which the mRNA was transcribed. Proteins are made up of one or more polypeptide molecules.

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2.10: Proteins

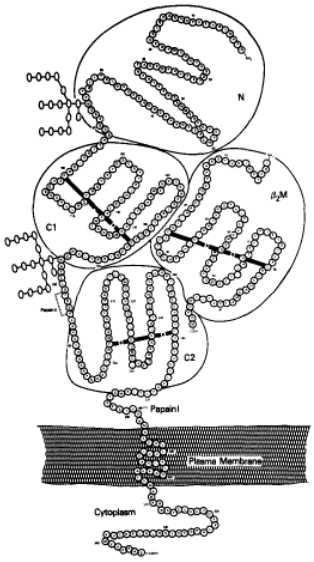
Proteins are macromolecules. They are constructed from one or more unbranched chains of amino acids; that is, they are polymers. An average eukaryotic protein contains around 500 amino acids but some are much smaller (the smallest are often called **peptides**) and some much larger (the largest to date is **titin** a protein found in skeletal and cardiac muscle; one version contains 34,350 amino acids in a single chain!).

Every function in the living cell depends on proteins.

- Motion and locomotion of cells and organisms depends on proteins. [Examples: Muscles, Cilia and Flagella]
- The catalysis of all biochemical reactions is done by **enzymes**, which contain protein.
- The structure of cells, and the extracellular matrix in which they are embedded, is largely made of protein. [Examples: Collagens] (Plants and many microbes depend more on carbohydrates, e.g., cellulose, for support, but these are synthesized by enzymes.)
- The transport of materials in body fluids depends of proteins.
- The receptors for hormones and other signaling molecules are proteins.
- Proteins are an essential nutrient for heterotrophs.
- The transcription factors that turn genes on and off to guide the differentiation of the cell and its later responsiveness to signals reaching it are proteins.
- and many more — proteins are truly the physical basis of life.

The protein represented here displays many of the features of proteins. Let's examine some of them as you scroll down the image. The protein consists of two polypeptide chains, a long one on the left of 346 amino acids — it is called the **heavy chain** — and a short one on the right of 99 amino acids. The heavy chain is shown as consisting of 5 main regions or **domains**:

- three extracellular domains, designated here as N (includes the **N-terminal**), C1, and C2;
- a transmembrane domain where the polypeptide chain passes through the plasma membrane of the cell;
- a cytoplasmic domain (with the **C terminal**) within the cytoplasm of the cell.



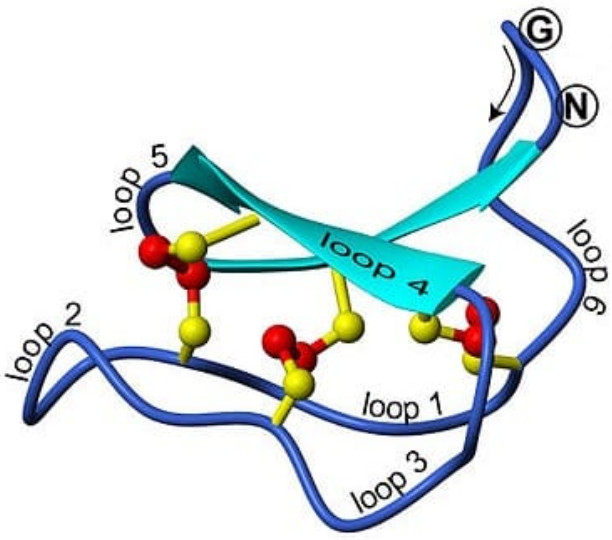


Figure 2.10.X: Structure of the prototypic cyclotide kalata B1 (public domain: KalataB1).

Inteins

Another, very rare, post-translational modification is the later removal of a section of the polypeptide and the splicing together (with a peptide bond) of the remaining N-terminal and C-terminal segments. The portion removed is called an **intein** (a "protein intron"), and the ligated segments are called **exteins** ("protein exons"). Genes encoding inteins have been discovered in a variety of organisms, including

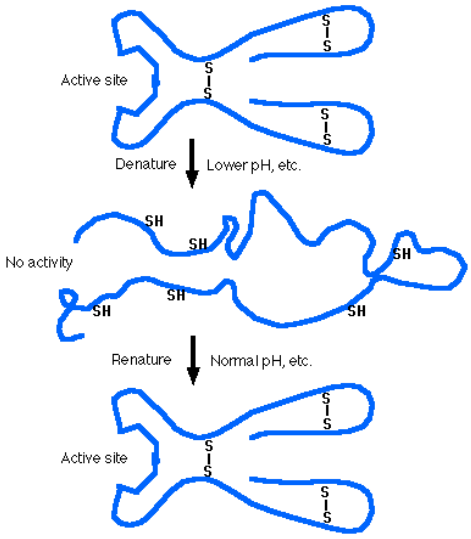
- some "true" bacteria such as
  - Bacillus subtilis
  - several mycobacteria
  - several blue-green algae (cyanobacteria)
- some Archaea such as
  - *Methanococcus jannaschii*
  - *Aeropyrum pernix*
- and a few unicellular eukaryotes, e.g., budding yeast (*Saccharomyces cerevisiae*).
- None has been found in the genomes of multicellular eukaryotes like Drosophila, *C. elegans*, or the green plant Arabidopsis.

How proteins get their shape

The function of a protein is determined by its shape. The shape of a protein is determined by its primary structure(sequence of amino acids). The sequence of amino acids in a protein is determined by the sequence of nucleotides in the gene (DNA) encoding it. The function of a protein (except when it is serving as food) is absolutely dependent on its three-dimensional structure. A number of agents can disrupt this structure thus **denaturing** the protein.

- changes in pH (alters electrostatic interactions between charged amino acids)
- changes in salt concentration (does the same)
- changes in temperature (higher temperatures reduce the strength of hydrogen bonds)
- presence of reducing agents (break S-S bonds between cysteines)

None of these agents breaks **peptide bonds**, so the primary structure of a protein remains intact when it is denatured. When a protein is denatured, it loses its function.



Example 2.10.1

- A denatured enzyme ceases to function.
- A denatured antibody no longer can bind its antigen.

Often when a protein has been **gently** denatured and then is returned to normal physiological conditions of temperature, pH, salt concentration, etc., it spontaneously regains its function (e.g. enzymatic activity or ability to bind its antigen). This tells us

- The protein has spontaneously resumed its native three-dimensional shape.
- Its ability to do so is **intrinsic**; no outside agent was needed to get it to refold properly.

However, there are:

- enzymes that add sugars to certain amino acids, and these may be essential for proper folding;
- proteins, called **molecular chaperones**, that may enable a newly-synthesized protein to acquire its final shape faster and more reliably than it otherwise would.

Chaperones

Although the three-dimensional (tertiary) structure of a protein is determined by its primary structure, it may need assistance in achieving its final shape.

- As a polypeptide is being synthesized, it emerges (N-terminal first) from the ribosome and the folding process begins.
- However, the emerging polypeptide finds itself surrounded by the watery cytosol and many other proteins.
- As hydrophobic amino acids appear, they must find other hydrophobic amino acids to associate with. Ideally, these should be their own, but there is the danger that they could associate with nearby proteins instead — leading to aggregation and a failure to form the proper tertiary structure.

To avoid this problem, the cells of all organisms contain molecular chaperones that stabilize newly-formed polypeptides while they fold into their proper structure. The chaperones use the energy of ATP to do this work.

Chaperonins

Some proteins are so complex that a subset of molecular chaperones — called chaperonins — is needed. Chaperonins are hollow cylinders into which the newly-synthesized protein fits while it folds. The inner wall of the cylinder is lined with hydrophobic amino acids which stabilize the hydrophobic regions of the polypeptide chain while it folds safely away from the

- watery cytosol and
- other proteins outside.

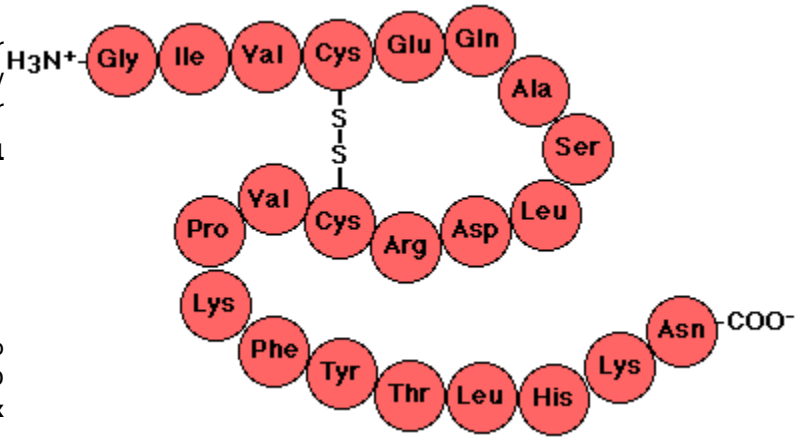
Chaperonins also use ATP as the energy source to drive the folding process.

As mentioned above, high temperatures can denature proteins, and when a cell is exposed to high temperatures, several types of molecular chaperones swing into action. For this reason, these chaperones are also called **heat-shock proteins** (HSPs). Not only do molecular chaperones assist in the folding of newly-synthesized proteins, but some of them can also unfold aggregated proteins and then refold the protein properly. Protein aggregation is the cause of disorders such as Alzheimer's disease, Huntington's disease, and prion diseases (e.g., "mad-cow" disease). Perhaps some day ways will be found to treat these diseases by increasing the efficiency of disaggregating chaperones.

Despite the importance of chaperones, the rule still holds: the final shape of a protein is determined by only one thing: the **precise sequence of amino acids** in the protein. And the sequence of amino acids in every protein is dictated by the sequence of nucleotides in the gene encoding that protein. So the function of each of the thousands of proteins in an organism is specified by one or more genes.

Primary Structure

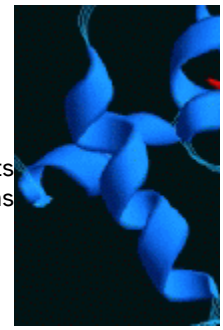
The primary structure of a protein is its linear sequence of amino acids and the location of any disulfide (-S-S-) bridges. Note the **amino terminal** or "**N-terminal**" (NH_3^+) at one end; **carboxyl terminal** ("**C-terminal**") (COO^-) at the other.



Secondary Structure

Most proteins contain one or more stretches of amino acids that take on a characteristic structure in 3-D space. The most common of these are the **alpha helix** and the **beta conformation**.

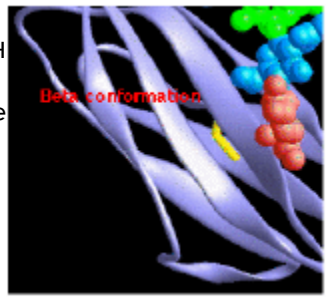
Alpha Helix



The R groups of the amino acids all extend to the outside.

- The helix makes a complete turn every 3.6 amino acids.
- The helix is right-handed; it twists in a clockwise direction.
- The carbonyl group (-C=O) of each peptide bond extends parallel to the axis of the helix and points directly at the -N-H group of the peptide bond 4 amino acids below it in the helix. A hydrogen bond forms between them [**-N-H·····O=C-**]

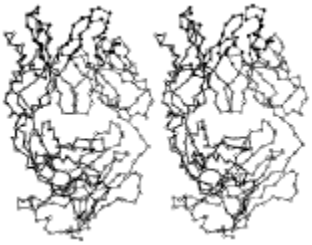
Beta Conformation



- consists of pairs of chains lying side-by-side and
- stabilized by hydrogen bonds between the carbonyl oxygen atom on one chain and the -NH group on the adjacent chain.
- The chains are often "anti-parallel"; the N-terminal to C-terminal direction of one being the reverse of the other.

Tertiary Structure

Tertiary structure refers to the three-dimensional structure of the entire polypeptide chain.



The images (courtesy of Dr. D. R. Davies) represent the tertiary structure of the antigen-binding portion of an antibody molecule. Each circle represents an alpha carbon in one of the two polypeptide chains that make up this protein. (The filled circles at the top are amino acids that bind to the antigen.) Most of the secondary structure of this protein consists of **beta conformation**, which is particularly easy to see on the right side of the image.

Do try to fuse these two images into a stereoscopic (3D) view. I find that it works best when my eyes are about 18" from the screen and I try to relax so that my eyes are directed at a point behind the screen.

Where the entire protein or parts of a protein are exposed to water (e.g., in blood or the cytosol), hydrophilic R groups — including R groups with sugars attached , are found at the surface; hydrophobic R groups are buried in the interior.

Importance of Tertiary structure

The function of a protein (except as food) depends on its tertiary structure. If this is disrupted, the protein is said to be **denatured**, and it loses its activity. Examples:

- denatured enzymes lose their catalytic power
- denatured antibodies can no longer bind antigen

A **mutation** in the gene encoding a protein is a frequent cause of altered tertiary structure.

- Curiously, tiny amounts of the mutant version can trigger the alpha-to-beta conversion in the normal protein. Thus the mutant version can be **infectious**. There have been several cases in Europe of people ill with Creutzfeldt-Jakob disease that may have acquired it from ingesting tiny amounts of the mutant protein in their beef.
- A number of other proteins altered by a point mutation in the gene encoding them, e.g.,
  - fibrinogen
  - lysozyme
  - transthyretin (a serum protein that transports thyroxin and retinol (vitamin A) in the blood)

  can form insoluble amyloid deposits in humans.

The many hydrogen bonds that can form between the polypeptide **backbones** in the beta conformation suggests that this is a stable secondary structure potentially available to many proteins and so a tendency to form insoluble aggregates is as well. Avoidance of amyloid formation may account for the large investment in the cell in

- chaperones
- proteasomes

as well as the crucial importance of particular amino acid side chains in maintaining a globular, and hence soluble, tertiary structure.

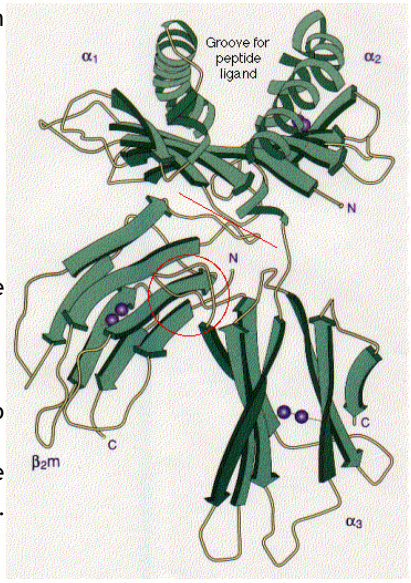
Protein Domains

The tertiary structure of many proteins is built from several **domains**. Often each domain has a separate function to perform for the protein, such as:

- binding a small ligand (e.g., a peptide in the molecule shown here)
- spanning the plasma membrane (transmembrane proteins)
- containing the catalytic site (enzymes)
- DNA-binding (in transcription factors)
- providing a surface to bind specifically to another protein

In some (but not all) cases, each domain in a protein is encoded by a separate exon in the gene encoding that protein. In the histocompatibility molecule shown here ,

- three domains α1, α2, and α3 are each encoded by its own exon.
- Two additional domains a transmembrane domain and a cytoplasmic domainare also encoded by separate exons.
- (β2-microglobulin, "β2m", is NOT a domain of this molecule. It is a separate molecule that binds to the three alpha domains (red line and circle) by noncovalent forces only. The complex of these two proteins is an example of quaternary structure.)



This image (courtesy of P. J. Bjorkman from Nature 329:506, 1987) is a schematic representation of the extracellular portion of HLA-A2, a human class I histocompatibility molecule. It also illustrates two common examples of secondary structure: the stretches of **beta conformation** are represented by the broad green arrows (pointing N -> C terminal); regions of **alpha helix** are shown as helical ribbons. The pairs of purple spheres represent the disulfide bridges. A correspondence between exons and domains is more likely to be seen in recently-evolved proteins. Presumably, "**exon shuffling**" during evolution has enabled organisms to manufacture new proteins, with new functions, by adding exons from other parts of the genome to encode new domains (rather like Lego® pieces).

Quaternary Structure

Complexes of 2 or more polypeptide chains held together by noncovalent forces (usually) but in precise ratios and with a precise 3-D configuration. The noncovalent association of a molecule of beta-2 microglobulin with the heavy chain of each class I histocompatibility molecule is an example.

Protein Kinesis

All proteins are synthesized by ribosomes using the information encoded in molecules of messenger RNA (**mRNA**). The various destinations for proteins occur in two major sets:

- one set for those proteins synthesized by **ribosomes** that remain suspended in the cytosol, and
- a second set for proteins synthesized by ribosomes that are attached to the membranes of the **endoplasmic reticulum** (**ER**) forming "rough endoplasmic reticulum" (RER).

Some of the important destinations for proteins are:

- the cytosol
- the nucleus
- mitochondria
- chloroplasts
- peroxisomes

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2.11: Rules of Protein Structure

The function of a protein is determined by its shape. The shape of a protein is determined by its primary structure (sequence of amino acids). The sequence of amino acids in a protein is determined by the sequence of nucleotides in the gene (DNA) encoding it. The function of a protein (except when it is serving as food) is absolutely dependent on its three-dimensional structure. A number of agents can disrupt this structure thus **denaturing** the protein.

- changes in pH (alters electrostatic interactions between charged amino acids)
- changes in salt concentration (does the same)
- changes in temperature (higher temperatures reduce the strength of hydrogen bonds)
- presence of reducing agents (break S-S bonds between cysteines)

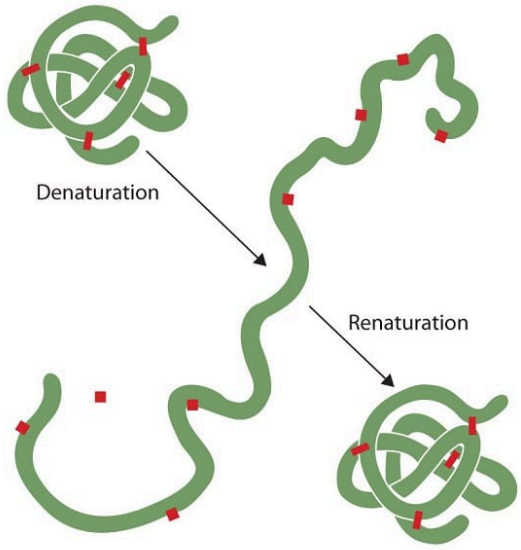


Figure 2.11.1: The denaturation (unfolding) and renaturation (refolding) of a protein is depicted. The red boxes represent stabilizing interactions, such as disulfide linkages, hydrogen bonding, and/or ionic bonds.

Often when a protein has been **gently** denatured and then is returned to normal physiological conditions of temperature, pH, salt concentration, etc., it spontaneously regains its function (e.g. enzymatic activity or ability to bind its antigen). This tells us that the protein has spontaneously resumed its native three-dimensional shape. Moreover, this ability is **intrinsic**; no outside agent was needed to get it to refold properly.

However, there are enzymes that add sugars to certain amino acids, and these may be essential for proper folding. These proteins, called **molecular chaperones**, enable a newly-synthesized protein to acquire its final shape faster and more reliably than it otherwise would.

Chaperones

Although the three-dimensional (tertiary) structure of a protein is determined by its primary structure, it may need assistance in achieving its final shape.

- As a polypeptide is being synthesized, it emerges (N-terminal first) from the ribosome and the folding process begins.
- However, the emerging polypeptide finds itself surrounded by the watery cytosol and many other proteins.
- As hydrophobic amino acids appear, they must find other hydrophobic amino acids to associate with. Ideally, these should be their own, but there is the danger that they could associate with nearby proteins instead — leading to aggregation and a failure to form the proper tertiary structure.

YouTube Video

This interactive element is not available in this format. Visit the link or scan the QR code to access it online.

View this YouTube Video online

Figure 11.2: Structure of the HSP90AA1 protein. from Wikipedia (CC_SA-BY-3.0 by Emw)

Despite the importance of chaperones, the rule still holds: the final shape of a protein is determined by only one thing: the **precise sequence of amino acids** in the protein. And the sequence of amino acids in every protein is dictated by the sequence of nucleotides in the gene encoding that protein. So the function of each of the thousands of proteins in an organism is specified by one or more genes.

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2.12: Glycoproteins

Glycoproteins have carbohydrate attached to them — a process called **glycosylation**. The attachment is a covalent linkage to:

- the hydroxyl (-OH) group of the R group of serine or threonine - called "**O-linked**" in both cases or to
- the amino group ($-NH_2$) in the R group of asparagine - called "**N-linked**"

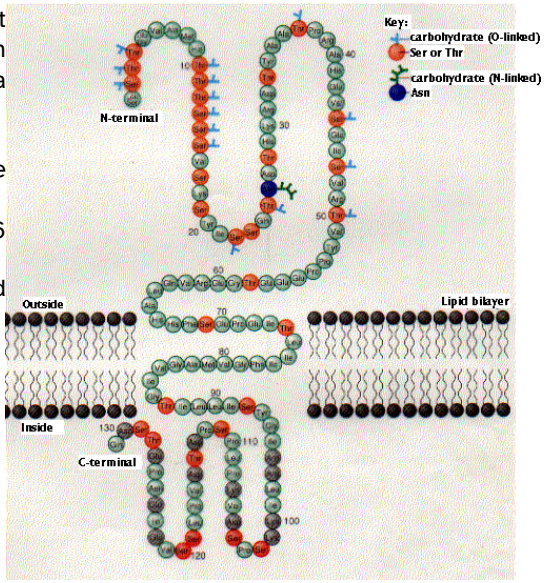
The carbohydrate consists of short, usually branched, chains of

- plain sugars (e.g., glucose, galactose)
- amino sugars (sugars with an amino group, e.g., N-acetylglucosamine), and
- acidic sugars (sugars with a carboxyl group, e.g., sialic acid)

Sugars are very hydrophilic thanks to their many -OH groups. Their presence

- makes glycoproteins far more hydrophilic than they would be otherwise and
- are often essential for the proper folding of the protein into its tertiary structure

Most of the proteins exposed to the watery surroundings at the surface of cells are glycoproteins.



This image shows the primary structure of **glycophorin A**, a glycoprotein that spans the plasma membrane ("Lipid bilayer") of human red blood cells. Each RBC has some 500,000 copies of the molecule embedded in its plasma membrane.

- Fifteen carbohydrate chains are "O-linked" to serine (Ser) and threonine (Thr) residues
- One carbohydrate chain is "N-linked" to the asparagine (Asn) at position 26

Two polymorphic versions of glycophorin A, which differ only at residues 1 and 5, occur in humans. These give rise to the MN blood groups

- The M allele encodes Ser at position 1 (Ser-1) and Gly at position 5 (Gly-5)
- The N allele encodes Leu-1 and Glu-5

Genotype to Phenotype

Individuals who inherit two N alleles (are homozygous) have blood group N.

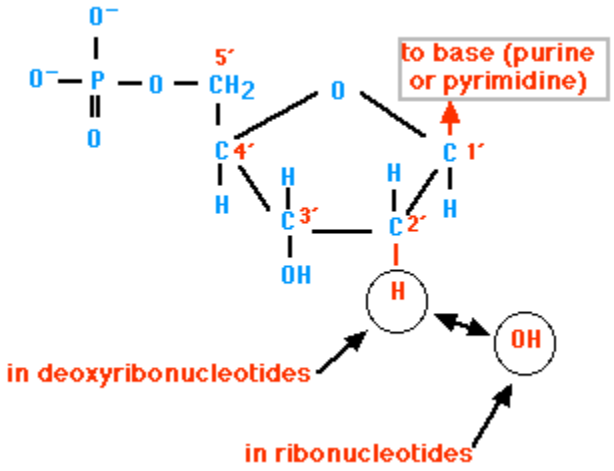
- Individuals who are homozygous for the M allele have blood group M.
- Heterozygous individuals produce both proteins and have blood group **MN**.

Glycophorin A is the most important attachment site by which the parasite *Plasmodium falciparum* invades human red blood cells.

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2.13: Nucleotides

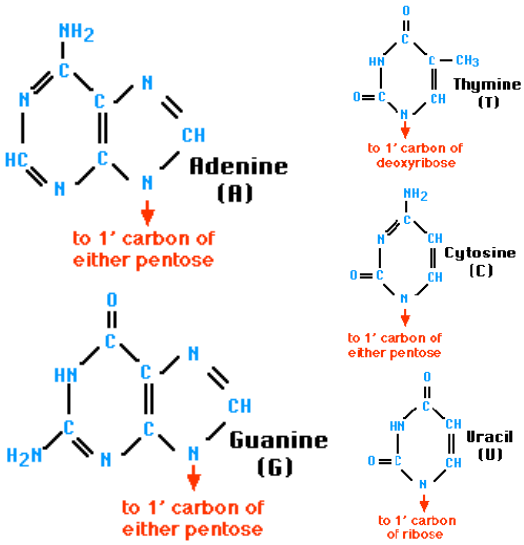
Nucleic acids are linear, unbranched polymers of **nucleotides**. Nucleotides consist of three parts.



A five-carbon sugar (hence a **pentose**). Two kinds are found:

- **deoxyribose**, which has a hydrogen atom attached to its #2 carbon atom (designated 2'), and
- **ribose**, which has a hydroxyl group there.

Deoxyribose-containing nucleotides, the **deoxyribonucleotides**, are the monomers of deoxyribonucleic acids (**DNA)**. Ribose-containing nucleotides, the **ribonucleotides**, are the monomers of ribonucleic acids (**RNA)**.



The Purines The Pyrimidines

A nitrogen-containing ring structure called a **nucleobase** (or simply a base). The nucleobase is attached to the 1' carbon atom of the pentose. In **DNA**, four different nucleobases are found:

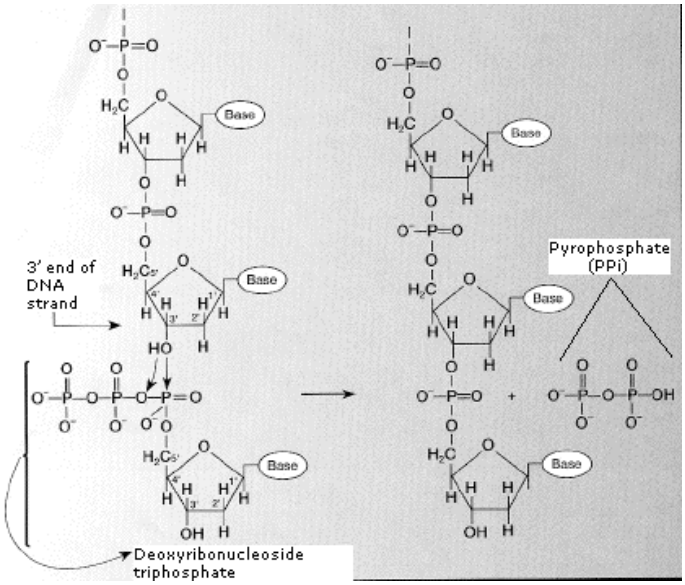
- two **purines**, called **adenine** (**A**) and **guanine** (**G**)
- two **pyrimidines**, called **thymine** (**T**) and **cytosine** (**C**)

RNA contains:

- The same purines, **adenine** (**A**) and **guanine** (**G**).
- RNA also uses the pyrimidine **cytosine** (**C**), but instead of thymine, it uses the pyrimidine **uracil** (**U**).

Nucleoside

The combination of a nucleobase and a pentose is called a **nucleoside**.



One (as shown in the first figure), two, or three **phosphate** groups. These are attached to the 5' carbon atom of the pentose. The product in each case is called a **nucleotide**. Both DNA and RNA are assembled from **nucleoside triphosphates**.

- For **DNA**, these are **dATP**, **dGTP**, **dCTP**, and **dTTP**.
- For **RNA**, these are **ATP**, **GTP**, **CTP**, and **UTP**.

In both cases, as each nucleotide is attached, the second and third phosphates are removed.

Table 2.13.1: The nucleosides and their mono-, di-, and triphosphates

	Nucleobase	**Nucleoside**	**Nucleotides**		
DNA	Adenine (A)	Deoxyadenosine	dAMP	dADP	dATP
	Guanine (G)	Deoxyguanosine	dGMP	dGDP	dGTP
	Cytosine (C)	Deoxycytidine	dCMP	dCDP	dCTP
	Thymine (T)	Deoxythymidine	dTMP	dTDP	dTTP
RNA	Adenine (A)	Adenosine	AMP	ADP	ATP
	Guanine (G)	Guanosine	GMP	GDP	GTP
	Cytosine (C)	Cytidine	CMP	CDP	CTP
	Uracil (U)	Uridine	UMP	UDP	UTP

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2.14: Proteomics

Genome

One complete set of genes in an organism (a haploid set).

Except for occasional unrepaired damage to its DNA (= mutations), the genome is fixed.

Transcriptome

The most common definition: All the messenger RNA (mRNA) molecules transcribed from the genome.

Varies with the differentiated state of the cell and the activity of the transcription factors that turn gene transcription on (and off).

Speaking strictly, one would define the transcriptome as **all** the RNA molecules — which includes a wide variety of untranslated, nonprotein-encoding RNA — transcribed from the DNA of the genome. It is now thought that ~75% of our DNA is transcribed into RNA although only 1.5% of this is messenger RNA for protein synthesis.

Metabolome

All the metabolic machinery, e.g.,

- enzymes
- coenzymes
- small metabolites, like
  - the intermediates in glycolysis and cellular respiration
  - nucleotides

present in a cell at a given time.

Varies with the differentiated state of the cell and its current activities.

Proteome

The proteome is the **prote**in complement of the gen**ome**. It is quite a bit more complicated than the genome because a **single gene** can give rise to a number of different proteins through

- alternative splicing of the pre-messenger RNAs (pre-mRNAs)
- RNA editing of the pre-messenger RNAs
- attachment of carbohydrate residues to form glycoproteins
- addition of phosphate groups to some of the amino acids in the protein

While we humans probably have only some 21 thousand genes, we probably make at least 10 times that number of different proteins. The great majority of our genes produce pre-mRNAs that are alternatively-spliced.

The study of proteomics is important because proteins are responsible for both the structure and the functions of all living things. Genes are simply the instructions for making proteins. It is proteins that make life.

The set of proteins within a cell varies

- from one differentiated cell type to another (e.g. red blood cell vs lymphocyte) and
- from moment to moment, depending on the activities of the cell, e.g.,
  - getting ready to duplicate its genome;

- repairing damage to its DNA;
- responding to a newly-available nutrient or cytokine;
- responding to the arrival of a hormone

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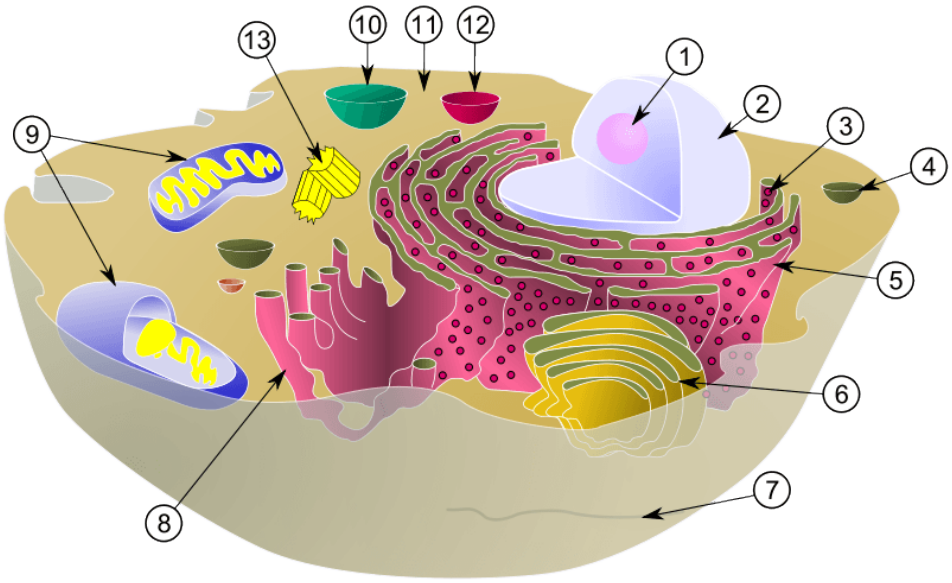
UNIT 3: THE CELLULAR BASIS OF LIFE

3.1: Animal Cells
3.2: Cell Membranes
3.3: The Nucleus
3.4: Ribosomes
3.5: Endoplasmic Reticulum
3.6: Golgi Apparatus
3.7: Centrosomes and Centrioles
3.8: Lysosomes and Peroxisomes
3.9: Protein Kinesis
3.10: The Proteasome
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3.12: Cilia
3.13: Animal Tissues
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3.15: Junctions between Cells
3.16: Plant Cells
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3.1: Animal Cells

This schematic represents an idealized animal cell, e.g., a liver cell. The columns to the left and right of the labels contain links to discussions of the particular structures.

Intermediate filaments Plasma membrane Peroxisome Vacuole Lysosome Nucleolus Centrioles Nucleus Nuclear envelope Golgi apparatus		Pinocytotic vesicle Actin filaments Glycogen granules Smooth endoplasmic reticulum Microtubules Ribosomes Mitochondrion Rough endoplasmic reticulum Cytosol

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3.2: Cell Membranes

One universal feature of all cells is an outer limiting membrane called the **plasma membrane**. In addition, all **eukaryotic cells** contain elaborate systems of **internal membranes** which set up various membrane-enclosed compartments within the cell. Cell membranes are built from **lipids** and **proteins**.

The Plasma Membrane

The plasma membrane serves as the interface between the machinery in the interior of the cell and the extracellular fluid (ECF) that bathes all cells. The lipids in the plasma membrane are chiefly phospholipids like phosphatidyl ethanolamine. Phospholipids are amphiphilic with the hydrocarbon tail of the molecule being hydrophobic; its polar head hydrophilic. As the plasma membrane faces watery solutions on both sides, its phospholipids accommodate this by forming a **phospholipid bilayer** with the hydrophobic tails facing each other. Substantial amounts of cholesterol are tucked within the hydrocarbon tails (not shown).

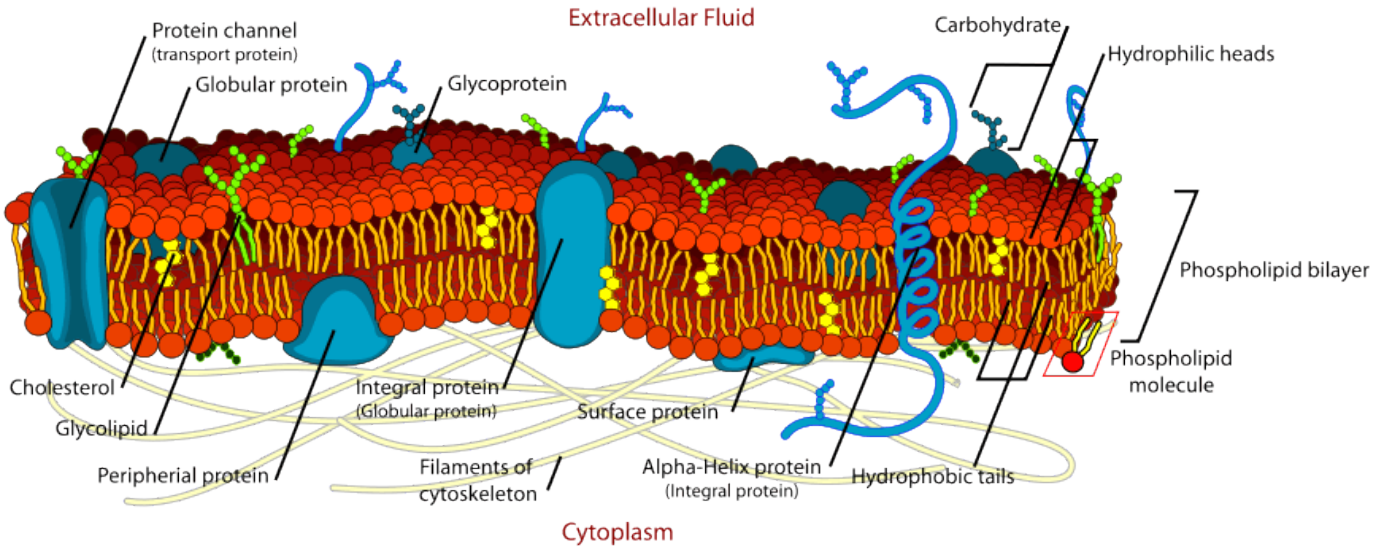


Figure 3.2.1: A detailed diagram of the cell membrane

Integral Membrane Proteins

Many of the proteins associated with the plasma membrane are tightly bound to it. Some are attached to lipids in the bilayer. In others — the **transmembrane proteins** — the polypeptide chain actually traverses the lipid bilayer (Figure 3.2.2). The figure shows a transmembrane protein that passes just once through the bilayer and another that passes through it 7 times. All G-protein-coupled receptors (e.g., receptors of peptide hormones, and odors) each span the plasma membrane 7 times.

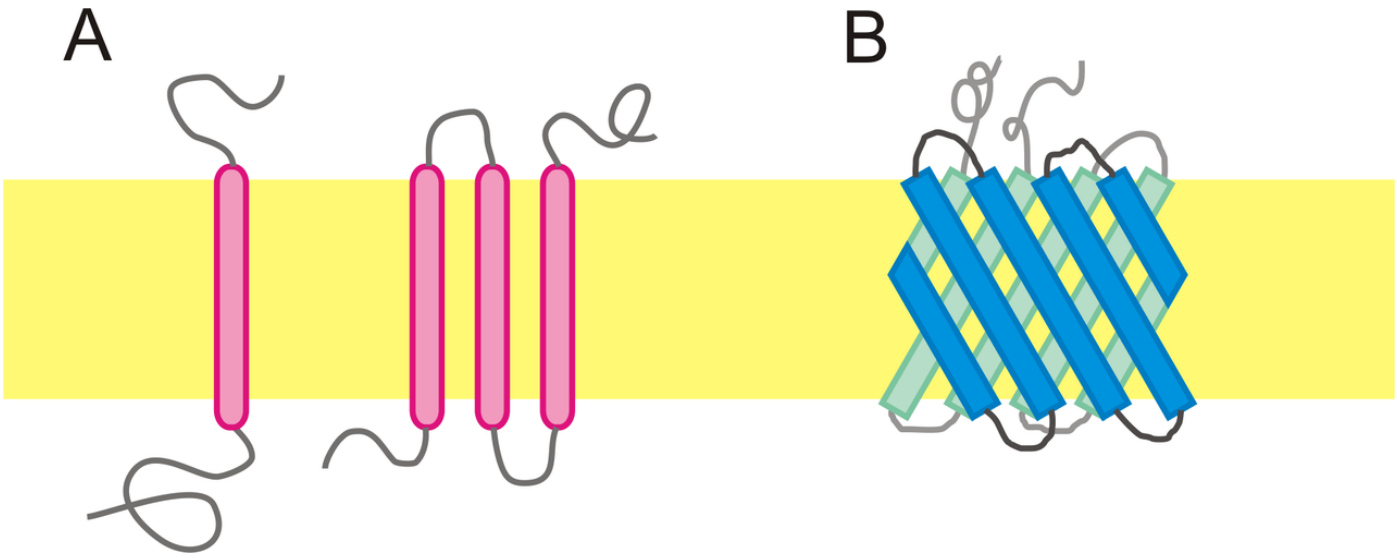


Figure 3.2.2: Schematic representation of transmembrane proteins: 1. a single transmembrane α-helix (bitopic membrane protein) 2. a polytopic transmembrane α-helical protein 3. a polytopic transmembrane β-sheet protein

In all these cases, the portion within the lipid bilayer consists primarily of hydrophobic amino acids. These are usually arranged in an alpha helix so that the polar -C=O and -NH groups at the peptide bonds can interact with each other rather than with their hydrophobic surroundings. Those portions of the polypeptide that project out from the bilayer tend to have a high percentage of hydrophilic amino acids. Furthermore, those that project into the aqueous surroundings of the cell are usually glycoproteins, with many hydrophilic sugar residues attached to the part of the polypeptide exposed at the surface of the cell. Some transmembrane proteins that span the bilayer several times form a hydrophilic channel through which certain ions and molecules can enter (or leave) the cell.

Peripheral Membrane Proteins

These are more loosely associated with the membrane. They are usually attached noncovalently to the protruding portions of integral membrane proteins (Figure 3.2.3). Membrane proteins are often restricted in their movements. A lipid bilayer is really a film of oil. Thus we might expect that structures immersed in it would be relatively free to float about. For some membrane proteins, this is the case. For others, however, their mobility is limited:

- Some of the proteins exposed at the interior face of the plasma membrane are tethered to cytoskeletal elements like actin microfilaments.
- Some proteins are the exterior face of the plasma membrane are anchored to components of the extracellular matrix like collagen.
- Integral membrane proteins cannot pass through the tight junctions found between some kinds of cells (e.g., epithelial cells).

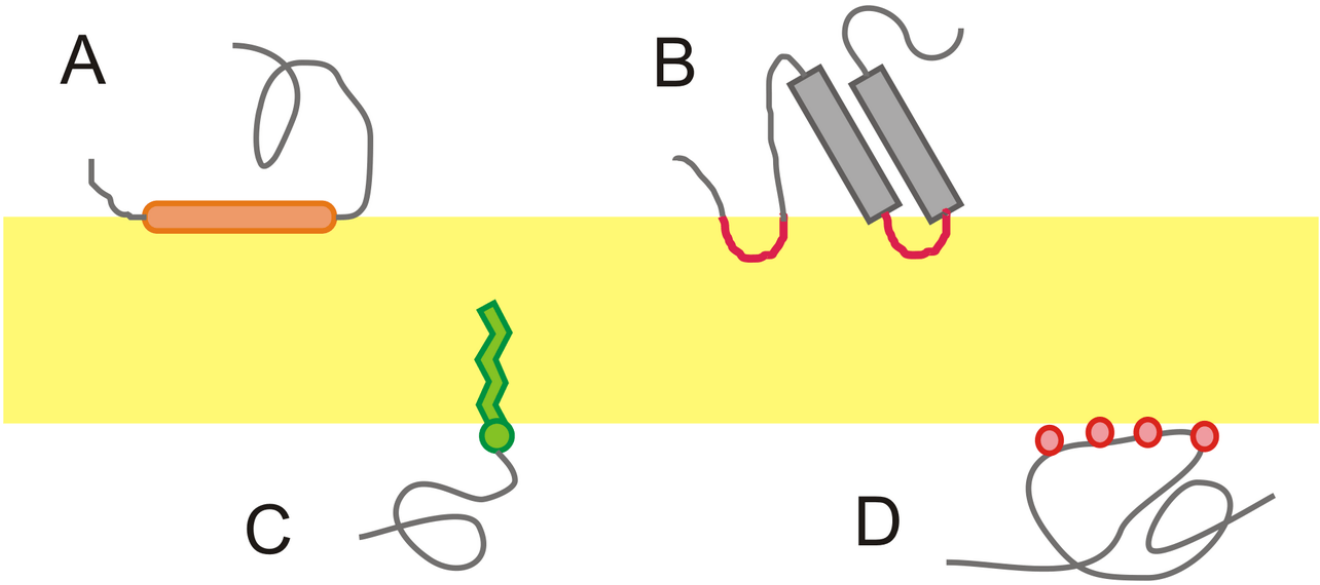


Figure 3.2.3: Schematic representation of peripheral membrane proteins

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3.3: The Nucleus

The nucleus is the hallmark of eukaryotic cells; the very term eukaryotic means having a "true nucleus".

The Nuclear Envelope

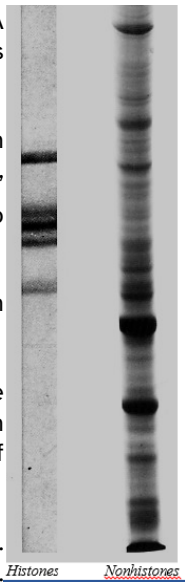
The nucleus is enveloped by a pair of membranes enclosing a lumen that is continuous with that of the endoplasmic reticulum. The inner membrane is stabilized by a meshwork of intermediate filament proteins called **lamins**. The nuclear envelope is perforated by thousands of **nuclear pore complexes** (**NPC**s) that control the passage of molecules in and out of the nucleus.

Chromatin

The nucleus contains the chromosomes of the cell. Each chromosome consists of a single molecule of DNA complexed with an equal mass of proteins. Collectively, the DNA of the nucleus with its associated proteins is called **chromatin**.

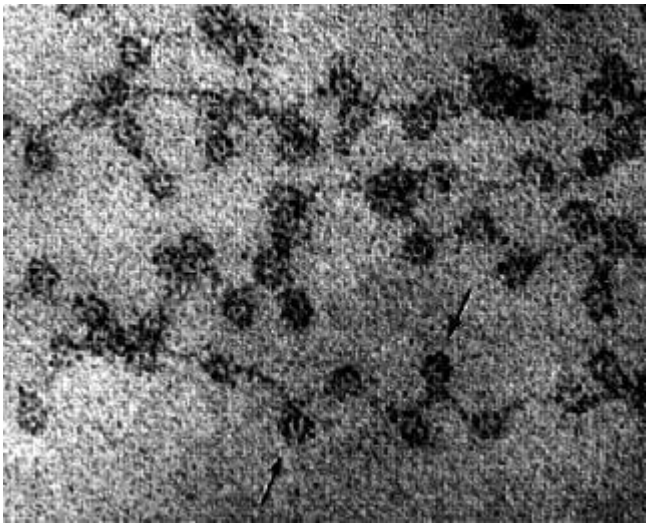
Most of the protein consists of multiple copies of 5 kinds of **histones**. These are basic proteins, bristling with positively charged arginine and lysine residues. (Both Arg and Lys have a free amino group on their R group, which attracts protons (H^+) giving them a positive charge.) Just the choice of amino acids you would make to bind tightly to the negatively-charged phosphate groups of DNA.

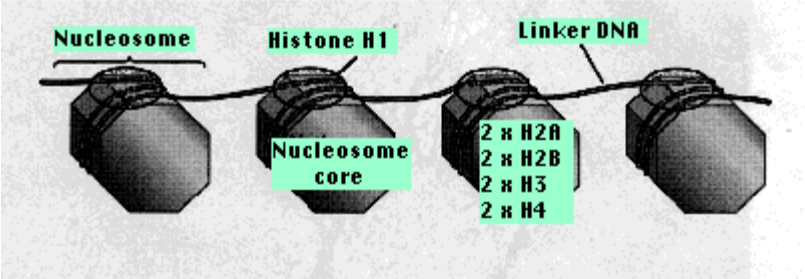
Chromatin also contains small amounts of a wide variety of **nonhistone proteins**. Most of these are transcription factors (e.g., the steroid receptors) and their association with the DNA is more transient.



The image on the right shows the 5 histones separated by electrophoresis. These 5 proteins vary little from one cell type to another or even from one species to another. However, the many nonhistone proteins in chromatin (also shown on the right) do vary from one cell type to another and from one species to another. (Courtesy of Gary S. Stein and Janet Swinehart Stein, University of Florida.)

Two copies of each of four kinds of histones H2A, H2B, H3, H4 form a core of protein, the **nucleosome** core. Around this is wrapped about 147 base pairs of DNA. From 20–60 bp of DNA link one nucleosome to the next. Each **linker** region is occupied by a single molecule of **histone 1** (**H1**). This region is longer (50–150 bp) adjacent to the promoters of genes which presumably makes more room for the binding of transcription factors.





Nucleosomes Nucleosome Schematics

The binding of histones to DNA does not depend on particular nucleotide sequences in the DNA but does depend critically on the amino acid sequence of the histone. Histones are some of the most conserved molecules during the course of evolution. Histone H4 in the calf differs from H4 in the pea plant at only 2 amino acids residues in the chain of 102. The above electron micrograph (courtesy of David E. Olins and Ada L. Olins) for nucleosomes shows chromatin from the nucleus of a chicken red blood cell (birds, unlike most mammals, retain the nucleus in their mature red blood cells). The arrows point to the nucleosomes. You can see why the arrangement of nucleosomes has been likened to "beads on a string".

The formation of nucleosomes helps somewhat, but not nearly enough, to make the DNA sufficiently compact to fit in the nucleus. In order to fit 46 DNA molecules (in humans), totaling over 2 meters in length, into a nucleus that may be only 10 µm across requires more extensive folding and compaction. Interactions between the exposed "tails" of the core histones causes nucleosomes to associate into a compact fiber 30 nm in diameter. These fibers are then folded into more complex structures whose precise configuration is uncertain and which probably changes with the level of activity of the genes in the region.

Histone Modifications

Although their amino acid sequence (primary structure) is unvarying, individual histone molecules do vary in structure as a result of chemical modifications that occur later to individual amino acids. These include adding:

- **acetyl** groups ($CH_3CO–$) to lysines
- phosphate groups to serines and threonines
- methyl groups to lysines and arginines

Although 75–80% of the histone molecule is incorporated in the core, the remainder — at the N-terminal — dangles out from the core as a "tail" (not shown in the figure). Most of the chemical modifications occur on these tails, especially of H3 and H4. Most of theses changes are reversible. For example, acetyl groups are **added** by enzymes called **histone acetyltransferases** (**HAT**s)(not to be confused with the "HAT" medium used to make monoclonal antibodies) and are also **removed** by **histone deacetylases** (**HDACs**). More often than not, acetylation of histones occurs in regions of chromatin that become active in gene transcription. This makes a kind of intuitive sense as adding acetyl groups neutralizes the positive charges on Lys thus reducing the strength of the association between the highly-negative DNA and the highly-positive histones.

However, there is surely more to the story. Acetylation of Lys-16 on H4 ("H4K16ac") prevents the interaction of their "tails" needed to form the compact 30-nm structure of inactive chromatin and thus is associated with active genes (note that this case involves interrupting protein-protein not protein-DNA interactions). Methylation, which also neutralizes the charge on lysines (and arginines), can either stimulate or inhibit gene transcription in that region.

- Adding 3 methyl groups to lysine-4 and/or lysine-36 in H3 (H3K4me3 and H3K36me3 respectively) is associated with **active** gene transcription while
- trimethylation of lysine-9 and/or lysine-27 in H3 (H3K9me3 and H3K27me3 respectively) is associated with **inactive** genes. (These include those **imprinted genes** that have been **permanently inactivated** in somatic cells.)
- And adding phosphates causes the chromosomes to become more — not less — compact as they get ready for mitosis and meiosis.

In any case, it is now clear that histones are a dynamic component of chromatin and not simply inert DNA-packing material.

Histone Variants

We have genes for 8 different varieties of histone 1 (H1). Which variety is found at a particular linker depends on such factors as the type of cell, where it is in the cell cycle, and its stage of differentiation. In some cases, at least, a particular variant of H1 associates with certain transcription factors to bind to the enhancer of specific genes turning off expression of those genes.

Some other examples of histone variants:

- H3 is replaced by **CENP-A** ("centromere protein A") at the nucleosomes near centromeres. Failure to substitute CENP-A for H3 in this regions blocks centromere structure and function.
- H2A is replaced by the variant H2A.Z at gene promoters and enhancers.
- All the "standard" histones are replaced by variants as sperm develop.

In general, the "standard" histones are incorporated into the nucleosomes as new DNA is synthesized during S phase of the cell cycle. Later, some are replaced by variant histones as conditions in the cell dictate.

Chromosome Territories

During interphase, little can be seen of chromatin structure (except for special cases like the polytene chromosomes of Drosophila and some other flies). Although each chromosome is greatly elongated, it tends to occupy a discrete region within the nucleus called its territory. This can be demonstrated by:

- directing a tiny laser beam at a small portion of the nucleus. If all the chromosomes were intertwined, one would expect that all would receive some damage. That does not occur — only one or two chromosomes are damaged.
- Fluorescent stains specific for a particular chromosome stain only two regions in the nucleus — revealing the territory of the two homologs.

"Kissing" Chromosomes

Portions of one chromosome can loop out of its territory and interact with part of a different chromosome looping out from its territory. These are "kissing" chromosomes. The examples that have been found so far indicate that these interactions are another way of coordinating the activity of genes residing on different chromosomes.

> The human genome contains many genes — scattered along different chromosomes — that are turned on by the arrival of a single signal. Among the many genes activated by estrogen, are *TIFF1* on chromosome 21 and *GREB1* on chromosome 2. Using FISH analysis, researchers at the University of California in San Diego showed that within a little as 2 minutes after exposing cells to estrogen, the *TIFF1* and *GREB1* loci move from their respective chromosome territories and "kiss".

> In the mouse, naive helper T cells — awaiting a signal to direct them to become either Th1 cells or Th2 cells have
>
> - the part of chromosome 10 carrying the gene for interferon-gamma (a Th1 cytokine) kissing
> - the part of chromosome 11 carrying the genes for IL-4 and IL-5 (Th2 cytokines).
>
> When the cell receives the signals committing it to one path or the other, the two regions separate, the appropriate one going to a region of active transcription; the other to a region of heterochromatin.

And still another example (in this case, two loci far apart on the **same** chromosome "kiss"):

In the head region of the Drosophila larva, expression of the homeobox (HOX) genes *Antp* and *Abd-B* is shut down. FISH analysis shows that these two loci — 10,000,000 base pairs apart on chromosome III — are brought together in the nucleus bound by proteins that prevent their transcription.

Euchromatin versus Heterochromatin

The density of the chromatin that makes up each chromosome (that is, how tightly it is packed) varies along the length of the chromosome. Dense regions are called heterochromatin and less dense regions are called euchromatin. Heterochromatin is found in parts of the chromosome where there are few or no genes, such as

- centromeres and
- telomeres. This heterochromatin is found in all types of cells in the organism.

- is also found in gene-rich regions of the chromosome but where the genes are inactive; that is, not transcribed. The location of this heterochromatin varies from one type of differentiated cell to another (as we would expect — a liver cell, for example, should shut down expression of genes that are not needed for its functions.
- is densely-packed.
- is greatly enriched with transposons and other types of DNA that does not contribute to the proteome.
- is replicated late in S phase of the cell cycle.
- has reduced crossing over in meiosis.
- is localized near the inner surface of the nuclear envelope, in most animal cells.
- The histones in the nucleosomes of heterochromatin show characteristic modifications:
  - **decreased** acetylation;
  - **increased** methylation of lysine-9 in histone H3 (H3K9), which now provides a binding site for **heterochromatin protein 1** (HP1), which blocks access by the transcription factors needed for gene transcription
  - **increased** methylation of lysine-27 in histone H3 (H3K27)

Euchromatin

- is found in parts of the chromosome that contain many active genes.
- is loosely-packed in loops of **30-nm fibers**.
- separated from adjacent **hetero**chromatin by **insulators**.
- In animal cells, euchromatin and thus active gene transcription occurs near the center of the nucleus.
- The genes in euchromatin show
  - **decreased methylation** of the cytosines in CpG sites of the gene's promoter(s)
  - **increased** acetylation of nearby histones
  - **decreased** methylation of lysine-9 and lysine-27 in histone H3

Nucleosomes and Transcription

Transcription factors cannot bind to their promoter if the promoter is blocked by a nucleosome. One of the first functions of the assembling transcription factors is to either expel the nucleosome from the site where transcription begins or at least to slide the nucleosomes along the DNA molecule. Either action exposes the gene's promoter so that the transcription factors can then bind to it.

The actual transcription of protein-coding genes is done by RNA polymerase II (Pol II or RNAP II). In order for it to travel along the DNA to be transcribed, a complex of proteins removes the nucleosomes in front of it and then replaces them after Pol II has transcribed that portion of DNA and moved on.

Nucleosomes and DNA Replication

As is the case in transcription, the DNA helix must open to allow DNA replication to proceed. This, too, requires that the nucleosomes preceding the replication fork be removed and then quickly reassembled as the leading and lagging strands are synthesized.

The Nucleolus

During the period between cell divisions, when the chromosomes are in their extended state, one or more of them (10 in human cells) have loops extending into a spherical mass called the nucleolus. Here are synthesized three (of the four) kinds of RNA

molecules (28S, 18S, 5.8S) used in the assembly of the large and small subunits of **ribosomes**.

28S, 18S, and 5.8S ribosomal RNA is transcribed (by RNA polymerase I) from hundreds to thousands of tandemly-arranged **rDNA genes** distributed (in humans) on 10 different chromosomes. The rDNA-containing regions of these 10 chromosomes cluster together in the nucleolus.

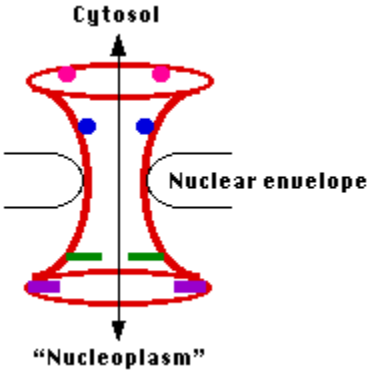
(In yeast, the 5S rRNA molecules — as well as transfer RNA molecules — are also synthesized (by RNA polymerase III) in the nucleolus.)

Once formed, rRNA molecules associate with the dozens of different ribosomal **proteins** used in the assembly of the large and small subunits of the ribosome.

But proteins are synthesized in the cytosol — and all the ribosomes are needed in the cytosol to do their work — so there must be a mechanism for the transport of these large structures in and out of the nucleus. This is one of the functions of the nuclear pore complexes.

Nuclear Pore Complexes (NPCs)

The nuclear envelope is perforated with thousands of pores. Each is constructed from multiple copies of several dozen different proteins called **nucleoporins**.



The entire assembly forms an aqueous channel connecting the cytosol with the interior of the nucleus ("nucleoplasm"). When materials are to be transported through the pore, it opens up to form a channel some 27–41 nm wide — large enough to get such large assemblies as ribosomal subunits through.

Transport through the nuclear pore complexes is **active**; that is, it requires

- energy
- many different carrier molecules each specialized to transport a particular cargo
- docking molecules in the NPC (represented here as colored rods and disks)

Import into the nucleus

Proteins are synthesized in the cytosol and those needed by the nucleus must be imported into it through the NPCs. They include:

- all the **histones** needed to make the nucleosomes
- all the **ribosomal proteins** needed for the assembly of ribosomes
- all the **transcription factors** (e.g., the steroid receptors) needed to turn genes on (and off)
- all the **splicing factors** needed to process pre-mRNA into mature mRNA molecules; that is, to cut out intron regions and splice the exon regions.

Probably all of these proteins has a characteristic sequence of amino acids — called a **nuclear localization sequence** (NLS) — that target them for entry.

Export from the nucleus

Molecules and macromolecular assemblies exported from the nucleus include:

- the **ribosomal subunits** containing both rRNA and proteins
- **messenger RNA** (mRNA) molecules (accompanied by proteins)
- **transfer RNA** (tRNA) molecules (also accompanied by proteins)
- **transcription factors** that are returned to the cytosol to await reuse

Both the RNA and protein molecules contain a characteristic **nuclear export sequence** (NES) needed to ensure their association with the right carrier molecules to take them out to the cytosol.

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3.4: Ribosomes

Ribosomes are the protein-synthesizing machines of the cell. They **translate** the information encoded in **messenger RNA** (**mRNA**) into a polypeptide.

Shape, size and function

Ribosomes are roughly spherical with a diameter of ~20 nm, they can be seen only with the electron microscope. Figure 3.4.1 is an electron micrograph showing clusters of ribosomes. These clusters, called polysomes, are held together by messenger RNA (mRNA). They can make up 25% of the dry weight of cells (e.g., pancreas cells) and specialize in protein synthesis. A single pancreas cell can synthesize 5 million molecules of protein per minute.

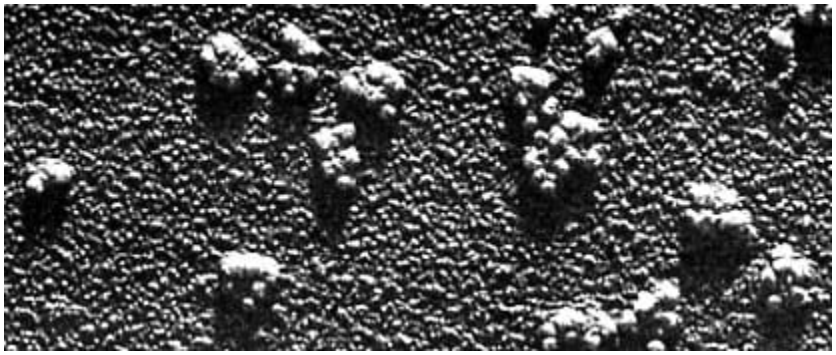


Figure 3.4.1: An electron micrograph of polysomes held together with mRNA. Image courtesy of Alexander Rich.

In eukaryotes, ribosomes that synthesize proteins for use within the cytosol (e.g., enzymes of glycolysis) are suspended in the cytosol. The specific ribosomes that synthesize proteins destined for secretion (by exocytosis), the plasma membrane (e.g., cell surface receptors), and lysosomes. These ribosomes are attached to the cytosolic face of the membranes of the endoplasmic reticulum. As the polypeptide is synthesized, it is extruded into the interior (lumen) of the endoplasmic reticulum. Then, before these proteins reach their final destinations, they undergo a series of processing steps in the Golgi apparatus.

Ribosomes that synthesize 13 of the proteins destined for the inner membrane of mitochondria are found within the mitochondrion itself and are quite different in structure from the others. The ribosomes of bacteria, eukaryotes, and mitochondria differ in many details of their structure (Table 3.4.1). However, despite these differences, the basic operations of bacterial, eukaryotic, and mitochondrial ribosomes are very similar.

Table 3.4.1: Comparison of Ribosome Structure in Bacteria, Eukaryotes, and Human Mitochondria

	Bacterial (70S)	Eukaryotic (80S)	Mitochondrial (55S)
Large Subunit	**50S**	**60S**	**39S**
rRNAs (1 of each)	23S (2904 nts)	28S (4700 nts)	16S (1560 nts)
	5S (120 nts)	5S (120 nts)	
		5.8S (160 nts)	
Proteins	35	47	50
Small Subunit	**30S**	**40S**	**28S**
rRNA	16S (1542 nts)	18S (1900 nts)	12S (950 nts)
Proteins	20	33	30
S values are the sedimentation coefficient: a measure of the rate at which the particles are spun down in the ultracentrifuge. S values are not additive. nts = nucleotides.			

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3.5: Endoplasmic Reticulum

The **endoplasmic reticulum** (ER) is a system of membrane-enclosed sacs and tubules in the cell. Their lumens are probably all interconnected, and their membranes are continuous with the outer membrane of the nuclear envelope. All the materials within the system are separated from the cytosol by a membrane. The endoplasmic reticulum is the site where the cell manufactures most of the membranes of the cell (plasma membrane, Golgi apparatus, lysosomes, nuclear envelope), lipids (including lipids for membranes, e.g., of the mitochondria, that are not made by the ER), and transmembrane proteins and secreted proteins. The ER comes in two versions: the **rough endoplasmic reticulum** (RER) and the **smooth endoplasmic reticulum** (SER).

Rough Endoplasmic Reticulum (RER)

The RER is typically arranged as interconnecting stacks of disc-like sacs. The cytosolic surface of the RER is studded with ribosomes engaged in protein synthesis. As the messenger RNA is translated by the ribosome, the growing polypeptide chain is inserted into the membrane of the RER. Proteins destined to be secreted by the cell or shipped into the lumen of certain other organelles like the Golgi apparatus and lysosomes pass all the way through into the lumen of the RER. Transmembrane proteins destined for the plasma membrane or the membrane of those organelles are retained within the membrane of the RER.

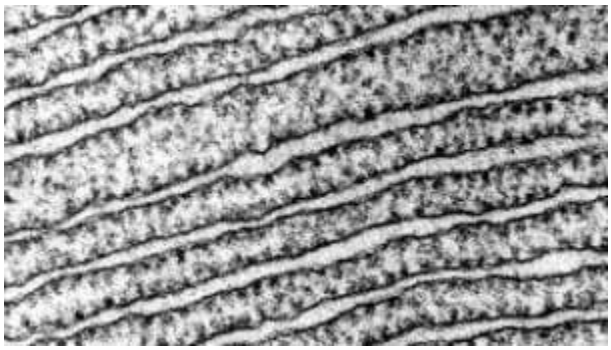


Figure 3.5.1: This electron micrograph (courtesy of Keith Porter) shows the RER in a bat pancreas cell. The clearer areas are the lumens.

In either case, the portion of the protein within the lumen of the RER is subject to extensive **glycosylation** (primarily N-linked) in Figure 3.5.1. The RER takes up a large proportion of the cytoplasm of cells specialized for protein synthesis such as cells secreting digestive enzymes (e.g. the pancreas cell above) and antibody-secreting plasma cells

Smooth Endoplasmic Reticulum (SER)

The SER differs from the RER in lacking attached ribosomes and usually being tubular rather than disc-like. A major function of the SER is the synthesis of lipids from which various cell membranes are made and like steroids, they are secreted from the cell. The SER represents only a small portion of the ER is most cells, e.g. serving as transport vesicles for the transport of protein to the Golgi apparatus. However, it is a prominent constituent of some cells, especially the cells of the adrenal cortex (which secrete steroid hormones), the cells of the liver (hepatocytes) where it synthesis lipids for secretion of lipoproteins, and the sarcoplasmic reticulum of muscle cells is SER.

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3.6: Golgi Apparatus

The Golgi apparatus is a cell structure mainly devoted to processing the proteins synthesized in the endoplasmic reticulum (ER). Some of these will eventually end up as integral membrane proteins embedded in the plasma membrane. Other proteins moving through the Golgi will end up in lysosomes or be secreted by exocytosis (e.g., digestive enzymes). The major processing activity is **glycosylation**: the adding of sugar molecules to form **glycoproteins**. In some cells, e.g., mucus-secreting cells in epithelia, the amount of carbohydrate so far exceeds that of the protein that the product is called a **mucopolysaccharide** (also known as a **proteoglycan**). In plant cells, the Golgi secretes the cell plate and cell wall.

Small peptides, e.g., some **hormones** and **neurotransmitters**, are typically too small to be synthesized directly by ribosomes. Instead, the ribosomes on the ER synthesize a large precursor protein that is later cut up into small peptide fragments as it traverses the Golgi. For example, proopiomelanocortin (POMC) is a polypeptide of 241 amino acids from which is cut ACTH, alpha and beta MSH, beta- endorphin, and others. POMC is cleaved to give rise to multiple peptide hormones.

- α-MSH produced by neurons in the arcuate nucleus has important roles in the regulation of appetite and sexual behavior, while α-MSH secreted from the intermediate lobe of the pituitary regulates the production of melanin.
- ACTH is a peptide hormone that regulates the secretion of glucocorticoids from the adrenal cortex.
- β-Endorphin and [Met]enkephalin are endogenous opioid peptides with widespread actions in the brain.

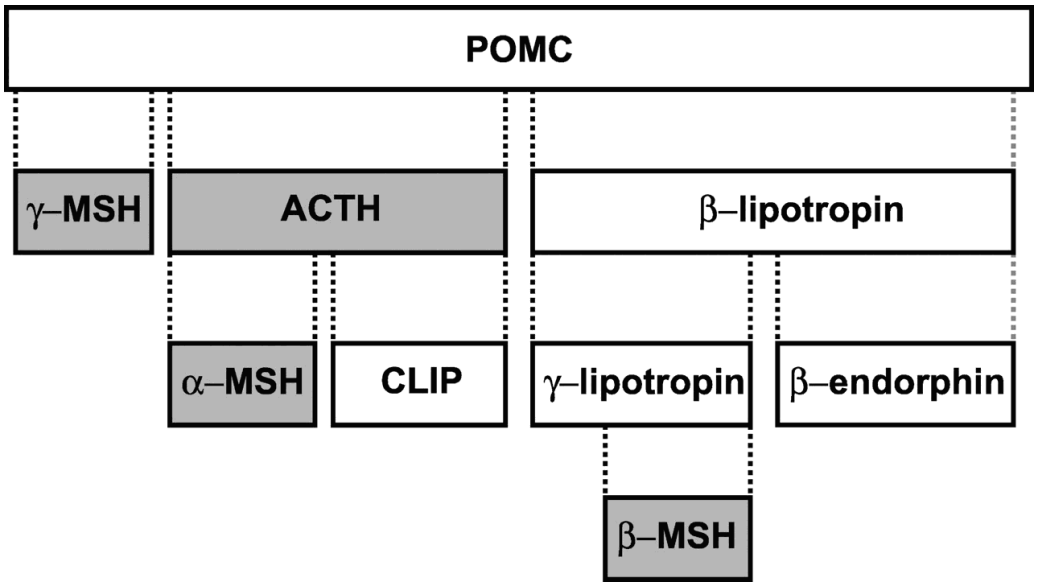


Figure 3.6.1: Proopiomelanocortin (POMC) and Melanocortin Peptides.

The Golgi consists of a stack of membrane-bounded **cisternae** located between the endoplasmic reticulum and the cell surface (Figure 3.6.2). Many different enzymes (proteins) are present in the Golgi to perform its various synthetic activities. So there must be mechanisms to sort out the processed proteins and send them on to their destinations while reclaiming processing proteins (e.g., glycosylases) for reuse.

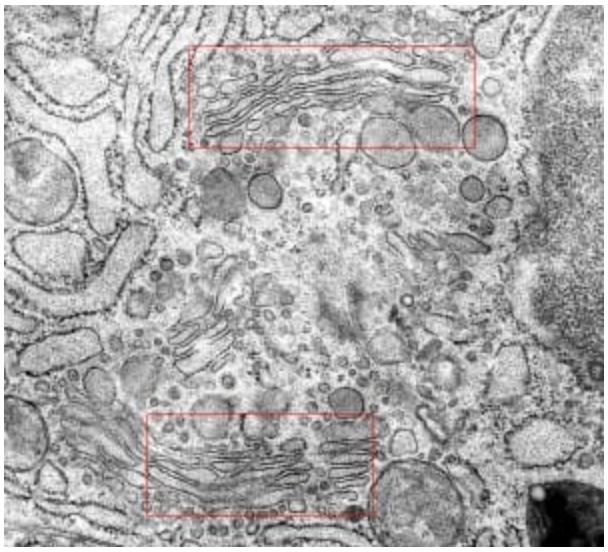


Figure 3.6.2: Golgi apparatus in a bat cell (Courtesy of Keith R. Porter)

The Outbound Path (Membrane Fission)

Two mechanisms appear to participate in the migration of proteins from the endoplasmic reticulum through the Golgi apparatus.

- **Mechanism 1:** Transition vesicles pinch off from the surface of the endoplasmic reticulum carrying integral membrane proteins, soluble proteins awaiting processing, and processing enzymes. Pinching off requires that the vesicle be coated with COPII (Coat Protein II). The transition vesicles move toward the *cis* Golgi on microtubules. As they do so, their COPII coat is removed and they may fuse together forming larger vesicles; these fuse with the *cis* Golgi. Sugars are added to proteins in small packets so many glycoproteins have to undergo a large number of sequential steps of glycosylation, each requiring its own enzymes. These steps take place as **shuttle vesicles** carry the proteins from ***cis*** to **medial** to the ***trans* Golgi** compartments. At the outer face of the *trans* Golgi, vesicles pinch off and carry their completed products to their various destinations.
- **Mechanism 2:** In addition to the pinching off and fusing of shuttle vesicles, the cisternae of the Golgi actually migrate themselves, that is, the *cis* Golgi gradually migrates up the stack becoming a medial and finally a *trans* Golgi (Figure 3.6.3).

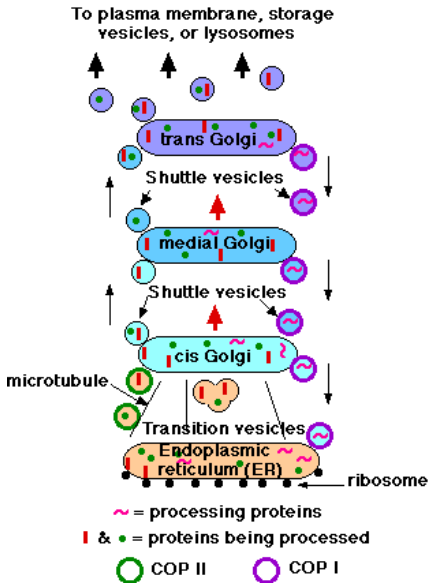


Figure 3.6.3

The Inbound Path (Membrane Fusion)

The movement of cisternal contents through the stack means that essential processing enzymes are also moving away from their proper site of action. Using a variety of signals, the Golgi separates the products from the processing enzymes that made them and returns the enzymes back to the endoplasmic reticulum. This transport is also done by pinching off vesicles, but the inbound vesicles are coated with **COPI** (**coat protein I**). A vesicle recognize its correct target by involving pairs of **complementary** integral membrane proteins:

- **v-SNAREs** = "vesicle SNAREs" — on the vesicle surface
- **t-SNAREs** = "target SNAREs" — on the surface of the target membrane

v-SNAREs and t-SNAREs bind specifically to each other thanks to the complementary structure of their surface domains. Binding is followed by fusion of the two membranes (Figure 3.6.4).

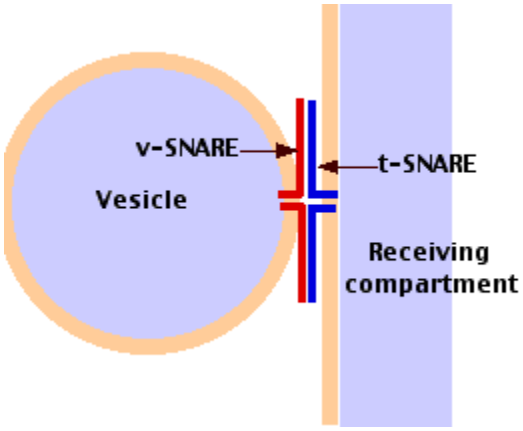


Figure 3.6.4

The Golgi is Not a Static Organelle

The Golgi breaks up and disappears at the onset of mitosis. By telophase of mitosis, the Golgi reappears. How it is recreated is still uncertain.

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3.7: Centrosomes and Centrioles

Centrioles

Centrioles are built from a cylindrical array of **9 microtubules**, each of which has attached to it **2 partial microtubules**. Figure 3.7.1 is an electron micrograph showing a cross section of a centriole with its array of nine triplets of microtubules.

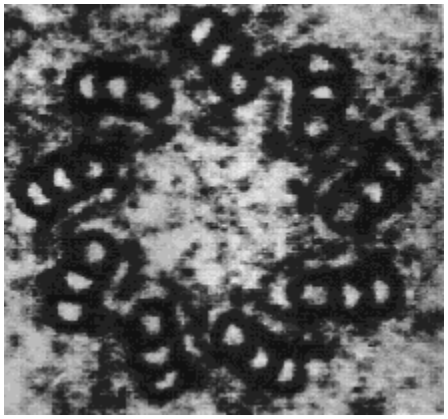


Figure 3.7.1: Cross Section of a Centriole (courtesy of E. deHarven). The magnification is approximately 305,000.

When a cell enters the cell cycle and passes through S phase, each centriole is duplicated. A "daughter" centriole grows out of the side of each parent ("mother") centriole. Thus centriole replication — like DNA replication (which is occurring at the same time) — is **semiconservative**.

- Functional microtubules grow out only from the "mother".
- When **stem cells** divide, one daughter cell remains a stem cell; the other goes on to differentiate. In two animal systems that have been examined (mouse glial cells and *Drosophila* male germline cells), the cell that receives the old ("mother") centriole remains a stem cell while the one that receives what had been the original "daughter" centriole goes on to differentiate. (You can read about these findings in Wang, X., *et. al.*, **Nature**, 15 October 2009.)

Centrioles are a key feature of eukaryotic cells and presumably arose with the first eukaryotes. A few groups have since lost their centrioles including most fungi (but not the primitive chytrids), "higher" plants (but not the more primitive mosses, ferns, and cycads with their motile sperm) and animal eggs lose their centriole during meiosis and must have it restored by the sperm that fertilizes it

In nondividing cells, the mother centriole can attach to the inner side of the plasma membrane forming a **basal body**. In almost all types of cell, the basal body forms a nonmotile primary cilium. In cells with a flagellum, e.g. sperm, the flagellum develops from a single basal body. (While sperm cells have a basal body, eggs have none. So the sperm's basal body is absolutely essential for forming a **centrosome** which will form a spindle enabling the first division of the zygote to take place.)

In **ciliated** cells such as the columnar epithelial cells of the lungs and ciliated protozoans like the paramecium, many basal bodies form, each producing a beating cilium. Most of their centrioles are produced by repeated duplication of the daughter centriole of centrosome and are temporarily assembled in a special organelle called the deutero**some** (not to be confused with deutero**stome**). Centrioles organize the **centrosome** in which they are embedded.

Centrosomes

The centrosome is located in the cytoplasm usually close to the nucleus. It consists of two **centrioles** — oriented at right angles to each other — embedded in a mass of amorphous material containing more than 100 different proteins.It is duplicated during S phase of the cell cycle. Just before mitosis, the two centrosomes move apart until they are on opposite sides of the nucleus. As mitosis proceeds, microtubules grow out from each centrosome with their plus ends growing toward the metaphase plate. These clusters of microtubules are called **spindle fibers**. Figure 3.7.2 shows microtubules growing in-vitro from an isolated centrosome.

The centrosome was supplied with a mixture of alpha and beta tubulin monomers spontaneously assembled into microtubules only in the presence of centrosomes.

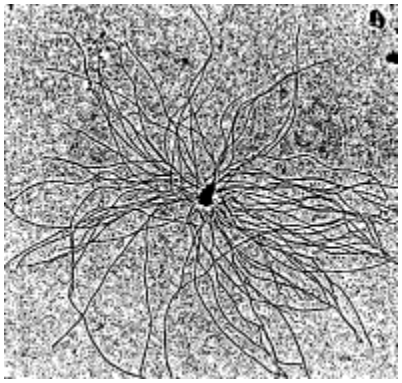


Figure 3.7.2: Centrosome (Courtesy Tim Hutchison)

Spindle fibers have three destinations:

1. Some attach to one **kinetochore** of a **dyad** with those growing from the opposite centrosome binding to the other kinetochore of that dyad.
2. Some bind to the arms of the chromosomes.
3. Still others continue growing from the two centrosomes until they extend between each other in a region of overlap.

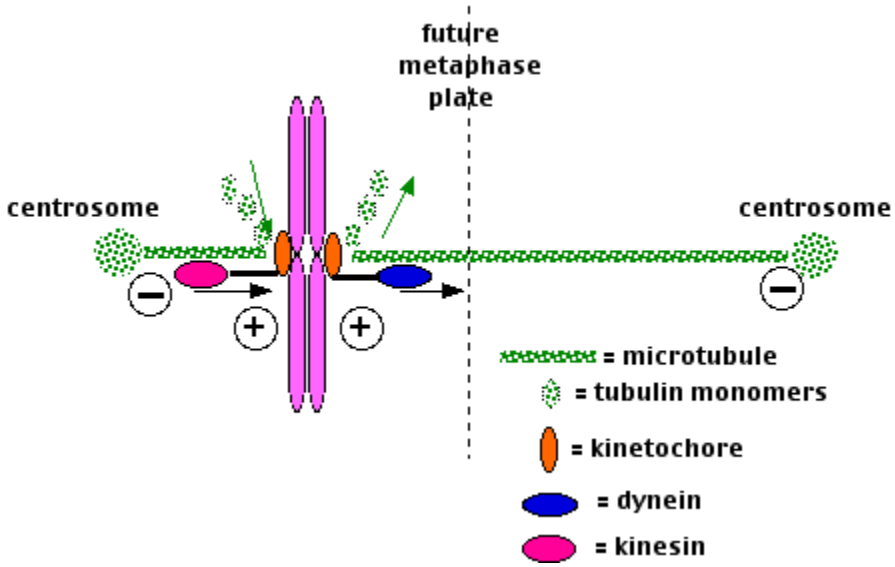


Figure 3.7.3: Chromosome assembly at metaphase

All three groups of **spindle fibers** participate in

- the assembly of the chromosomes at the metaphase plate at metaphase.

    1. Microtubules attached to opposite sides of the dyad shrink or grow until they are of equal length.
    2. Microtubules motors attached to the kinetochores move them
        - toward the **minus** end of **shrinking** microtubules (a **dynein**);
        - toward the **plus** end of **lengthening** microtubules (a **kinesin**).
    3. The chromosome **arms** use a different **kinesin** to move to the metaphase plate.

- the separation of the chromosomes at anaphase.
    1. The sister kinetochores separate and, carrying their attached chromatid,
    2. move along the microtubules powered by minus-end motors, **dyneins**, while the microtubules themselves shorten (probably at both ends).

3. The overlapping spindle fibers move past each other (pushing the poles farther apart) powered by plus-end motors, the "bipolar" **kinesins**.
4. In this way the sister chromatids end up at opposite poles.

Functions of Centrosomes

In addition to their role in spindle formation, centrosomes play other important roles in animal cells:

- Formation of the network of microtubules that participate in making the cytoskeleton.
- Signaling that it is o.k. to proceed to cytokinesis. Destruction of both centrosomes with a laser beam prevents cytokinesis even if mitosis has been completed normally.
- Signaling that it is o.k. for the daughter cells to begin another round of the cell cycle; specifically to duplicate their chromosomes in the next **S phase**. Destruction of one centrosome with a laser beam still permits cytokinesis but the daughter cells fail to enter a new S phase.
- Segregating signaling molecules (e.g., mRNAs) so that they pass into only one of the two daughter cells produced by mitosis. In this way, the two daughter cells can enter different pathways of differentiation even though they contain identical genomes.
- In at least some developing neurons, the position of the centrosome establishes the point at which the axon will grow out.

Centrosomes and Cancer

Cancer cells often have more than the normal number of centrosomes. They also are aneuploid (have abnormal numbers of chromosomes), and considering the role of centrosomes in chromosome movement, it is tempting to think that the two phenomena are related. Mutations in the tumor suppressor gene *p53* seem to predispose the cell to excess replication of the centrosomes. Chromosome movement in mitosis also involves polymerization and depolymerization of microtubules.

Because the hallmark of cancer cells is uncontrolled mitosis, both vincristine and Taxol are used as anticancer drugs. **Vincristine**, a drug found in the Madagascar periwinkle (a wildflower), binds to tubulin dimers preventing the assembly of microtubules. This halts cells in metaphase of mitosis and **Taxol**, a drug found in the bark of the Pacific yew, prevents depolymerization of the microtubules of the spindle fiber. This, in turn, stops chromosome movement, and thus prevents the completion of mitosis.

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3.8: Lysosomes and Peroxisomes

A cell is composed of many different organelles and microbodies (or cytosomes) is a type of organelle that is found in the cells of plants, protozoa, and animals. Organelles in the microbody family include peroxisomes, glyoxysomes, glycosomes and hydrogenosomes.

Lysosomes

Lysosomes are roughly spherical bodies enclosed by a single membrane. They are manufactured by the Golgi apparatus (Figure 3.8.1) and contain over 50 different kinds of hydrolytic enzymes including proteases, lipases, nucleases, and polysaccharidases. The pH within the lysosome is about pH 5, substantially less than that of the cytosol (~pH 7.2). All the enzymes in the lysosome work best at an acid pH, which reduces the risk of their digesting their own cell if they should escape from the lysosome.

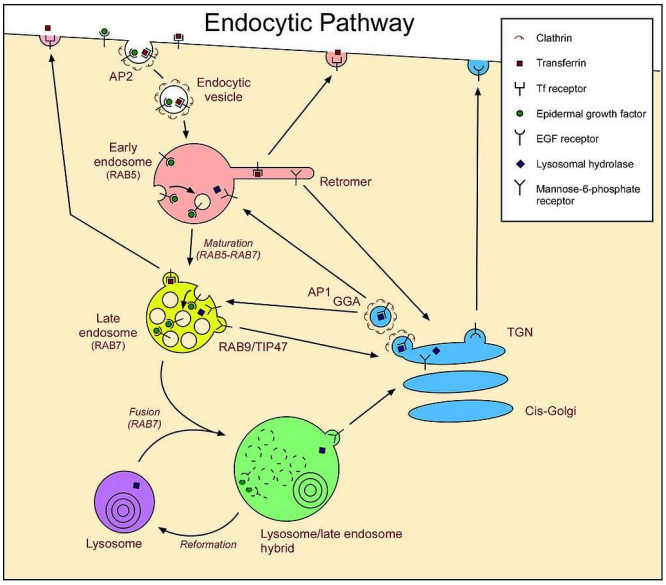


Figure 3.8.1**:** *Lysosome manufacturing process The lysosome is shown in purple, as an endpoint in Endocytotic sorting. AP2 is necessary for vesicle formation, whereas the Mannose-6-receptor is necessary for sorting Hydrolase into the Lysosome's lumen. Image used wtih permission (CC BY-SA; Matthew R G Russell).*

Materials within the cell scheduled for digestion are first deposited within lysosomes. These may be:

- other organelles, such as mitochondria, that have ceased functioning properly and have been engulfed in **autophagosomes**
- food molecules or, in some cases, food particles taken into the cell by endocytosis
- foreign particles like bacteria that are engulfed by neutrophils
- antigens that are taken up by
- "professional" antigen-presenting cells like dendritic cells (by phagocytosis) and
- B cells (by binding to their antigen receptors (BCRs) followed by receptor-mediated endocytosis.

> At one time, it was thought that lysosomes were responsible for killing cells scheduled to be removed from a tissue; for example, the resorption of its tail as the tadpole metamorphoses into a frog. This is incorrect. These examples of programmed cell death (PCD) or apoptosis take place by an entirely different mechanism.

In some cells, lysosomes have a secretory function — releasing their contents by exocytosis.

- Cytotoxic T cells (CTL) secrete perforin from lysosomes.
- Mast cells secrete some of their many mediators of inflammation from modified lysosomes.
- Melanocytes secrete melanin from modified lysosomes.
- The exocytosis of lysosomes provides the additional membrane needed to quickly **seal wounds** in the plasma membrane.

Lysosomal Storage Diseases

Lysosomal storage diseases are caused by the accumulation of macromolecules (proteins, polysaccharides, lipids) in the lysosomes because of a genetic failure to manufacture an enzyme needed for their breakdown. Neurons of the central nervous system are particularly susceptible to damage. Most of these diseases are caused by the inheritance of two defective alleles of the gene encoding **one** of the hydrolytic enzymes. Examples include:

- **Tay-Sachs disease** and **Gaucher's disease** — both caused by a failure to produce an enzyme needed to break down sphingolipids (fatty acid derivatives found in all cell membranes).
- **Mucopolysaccharidosis I** (MPS-I). Caused by a failure to synthesize an enzyme (α-L-iduronidase) needed to break down proteoglycans like heparan sulfate. In April 2003, the U.S. Food and Drug Administration approved a synthetic version of the enzyme, laronidase (Aldurazyme®), as a possible treatment. This enzyme (containing 628 amino acids) is manufactured by recombinant DNA technology.

However, one lysosomal storage disease, **I-cell disease** ("inclusion-cell disease"), is caused by a failure to "tag" (by phosphorylation) **all** the hydrolytic enzymes that are supposed to be transported from the Golgi apparatus to the lysosomes. Lacking the mannose 6-phosphate (M6P) tag, they are secreted from the cell instead. The result: all the macromolecules incorporated in lysosomes remain undegraded forming "inclusion bodies" in the cell.

Peroxisomes

Peroxisomes, also called microbodies, are about the size of lysosomes (0.5–1.5 μm) and like them are enclosed by a single membrane. They also resemble lysosomes in being filled with enzymes. However, peroxisomes bud off from the endoplasmic reticulum, not the Golgi apparatus (the source of lysosomes) and the enzymes and other proteins destined for peroxisomes are synthesized in the cytosol. Each contains a **p**eroxisomal **t**argeting **s**ignal (**PTS**) that binds to a receptor molecule that takes the protein into the peroxisome and then returns for another load. Two peroxisomal targeting signals have been identified: a 9-amino acid sequence at the N-terminal of the protein and a tripeptide at the C-terminal. Each has its own receptor to take it to the peroxisome.

Functions of the peroxisomes in the human liver inlcude:

- Breakdown (by oxidation) of excess fatty acids.
- Breakdown of hydrogen peroxide (H_2O_2), a potentially dangerous product of fatty-acid oxidation. It is catalyzed by the enzyme catalase.
- Participates in the synthesis of cholesterol. One of the enzymes involved, HMG-CoA reductase, is the target of the popular cholesterol-lowering "statins".
- Participates in the synthesis of bile acids.
- Participates in the synthesis of the lipids used to make myelin.
- Breakdown of excess purines (AMP, GMP) to uric acid.

Peroxisomes are also present in plant cells where they participate is such functions as symbiotic nitrogen fixation and photorespiration.

Peroxisome Disorders

A variety of rare inherited disorders of peroxisome function occur in humans. Most involve mutant versions of one or another of the enzymes found within peroxisomes. For example: **X-linked adrenoleukodystrophy** (**X-ALD**) results from a failure to metabolize fatty acids properly. One result is deterioration of the myelin sheaths of neurons. The disorder occurs in young boys because the gene is X-linked. An attempt to find an effective treatment was the subject of the 1992 film **Lorenzo's Oil**. A few diseases result from failure to produce functional peroxisomes. For example: **Zellweger syndrome** results from the inheritance of two mutant genes for one of the receptors (PXR1) needed to import proteins into the peroxisome.

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3.9: Protein Kinesis

Proteins are the major building blocks of life. Eukaryotic cells synthesize proteins for thousands of different functions. Some examples:

- to build the components of the cytosol (e.g. microtubules, glycolytic enzymes);
- to build the receptors and other molecules exposed at the surface of the cell embedded in the plasma membrane;
- to supply some of the components of the mitochondria and (in plant cells) chloroplasts;
- proteins secreted from the cell to supply the needs of other cells and tissues (e.g. collagens to support cells, hormones to signal them).

All proteins are synthesized by ribosomes using the information encoded in molecules of messenger RNA (**mRNA**). This process is called translation and is described in Gene Translation: RNA -> Protein. Our task here is to explore the ways that these proteins are delivered to their proper destinations.

The various destinations for proteins occur in two major sets: (1) one set for those proteins synthesized by **ribosomes** that remain suspended in the cytosol, and (2) a second set for proteins synthesized by ribosomes that are attached to the membranes of the endoplasmic reticulum (ER) forming "rough endoplasmic reticulum" (RER). This electron micrograph (courtesy of Keith Porter) shows the RER in a bat pancreas cell. The clearer areas are the lumens. So the first decision that must be made as a ribosome begins to translate a mRNA into a polypeptide is whether to remain free in the cytosol or to bind to the ER.

Pathways Through the Endoplasmic Reticulum (ER)

The decision to enter the ER is dictated by the presence of a **signal sequence** on the growing polypeptide. The signal sequence consists of the first portion of the elongating polypeptide chain (so the signal sequence occurs at the amino terminal of the polypeptide). Typical signal sequences contain 15–30 amino acids. The precise amino acid sequence varies surprisingly from one protein to the next, but all signal sequences include many hydrophobic amino acids.

If a signal sequence **is** present,

- translation ceases after it has been synthesized.
- The signal sequence is recognized by and is bound by a **signal recognition particle** (**SRP**).
- The complex of ribosome with its nascent polypeptide and the SRP binds to a receptor on the surface (facing the cytosol) of the ER.
- The SRP leaves and translation recommences.
- The growing polypeptide chain is extruded through a pore in the ER membrane and into the **lumen** of the ER.
- The signal sequence is usually clipped off the polypeptide unless the polypeptide is to be retained as an integral membrane protein.
- Other proteins, called **molecular chaperones**, present in the lumen of the ER, bind the growing polypeptide chain and assist it to fold into its correct tertiary structure.
- Sugar residues may be added to the protein. The process is called **glycosylation** and often is essential for proper folding of the final product, a glycoprotein.

> **Note**
>
> The 1999 Nobel Prize in Physiology or Medicine was awarded to Dr. Günter Blobel for his discovery of the signal sequence and other intrinsic signals that enable proteins to reach their proper destinations.

Destinations of proteins synthesized within the ER

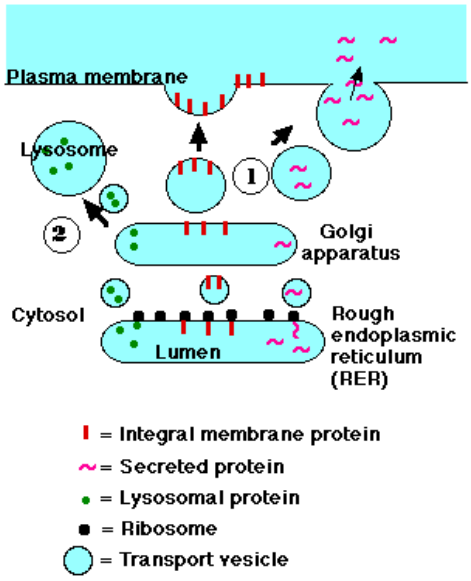


Figure 3.9.1: Glycosylation

There are two options.

1. proteins glycosylated with residues of mannose-6-phosphate will leave the Golgi in transport vesicles that eventually fuse with **lysosomes** (path **2** in fig. 3.9.1).
2. proteins that do not receive this marker, leave in transport vesicles that eventually fuse with the **plasma membrane** (path **1** in fig. 3.9.1). These are integral membrane proteins that become exposed at the surface of the cell (forming receptors and the like) and proteins in solution within the transport vesicle. These are discharged from the cell. This secretory process is called **exocytosis**.

The Signal Recognition Particle (SRP)

The signal recognition particle in mammalian cells is made from:

- a single small (7S) molecule of RNA
- six different molecules of protein

It contains binding sites for the signal sequence, the ribosome, and an SRP receptor, also called the docking protein, on the cytosol face of the membranes of the ER.

Destinations of Proteins Synthesized By Free Ribosomes

Ribosomes synthesizing a protein without a signal sequence do not bind to the ER and continue synthesis until the polypeptide is completed. Chaperones are also present in the cytosol that help the protein assume its final three-dimensional configuration. Some of the important destinations for these proteins are:

- The **cytosol** itself. Such proteins as the enzymes of glycolysis, tubulins for making microtubules, and actin for making microfilaments are simply released from the ribosome and go to work.
- The **nucleus**. Many proteins — **histones**, **transcription factors**, and ribosomal proteins are notable examples — must move from the cytosol into the interior of the nucleus. They are targeted to the nucleus by their **nuclear localization sequence**, a sequence of 7–41 amino acids of which the basic amino acids lysine and arginine are characteristic members. These proteins are actively transported through pores in the nuclear envelope into the interior.
- Mitochondria. Although the mitochondrion has its own genome and protein-synthesizing machinery, most of the proteins used by mitochondria are Proteins destined for mitochondrion contain a characteristic signal sequence. This is recognized and bound by a **chaperone** called **mitochondrial stimulation factor** (**MSF**). MSF targets the protein to a receptor embedded in the outer membrane of the mitochondrion. Other factors and receptors shepherd proteins through the intermembrane

space to the inner mitochondrial membrane (e.g. some proteins of the electron transport chain) and the matrix.
    - encoded by genes in the nucleus of the cell
    - synthesized in the cytosol
    - must be imported into the mitochondrion.
- **Chloroplasts**. Chloroplasts, like mitochondria, have their own genome and their own protein-synthesizing machinery. But also like mitochondria, most of the proteins used in chloroplasts are encoded by genes in the nucleus of the cell, are synthesized by ribosomes in the cytosol, and must then be imported into the chloroplast. Proteins destined for chloroplasts are recognized by their characteristic **transit sequence**. Chaperones are also needed to get them to their final destination: stroma, thylakoid membrane, etc.
- **Peroxisomes**. Proteins destined for peroxisomes are synthesized with a **p**eroxisomal **t**argeting **s**ignal (**PTS**) that binds to a receptor molecule that takes the protein into the peroxisome and then returns for another load.

  Two peroxisomal targeting signals have been identified:

  Each has its own receptor to take it to the peroxisome.
    - a 9-amino acid sequence at the N-terminal of the protein
    - a tripeptide at the C-terminal.

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3.10: The Proteasome

Protein **degradation** is as essential to the cell as protein **synthesis**. For example, to supply amino acids for fresh protein synthesis, to remove excess enzymes, and to remove transcription factors that are no longer needed. There are two major intracellular devices in which damaged or unneeded proteins are broken down. They are **lysosomes** and **proteasomes**

Lysosomes

Lysosomes deal primarily with **extracellular** proteins, e.g., plasma proteins, that are taken into the cell, e.g., by endocytosis. They are cell-surface membrane proteins that are used in receptor-mediated endocytosis. The proteins (and other macromolecules) are engulfed by **autophagosomes.**

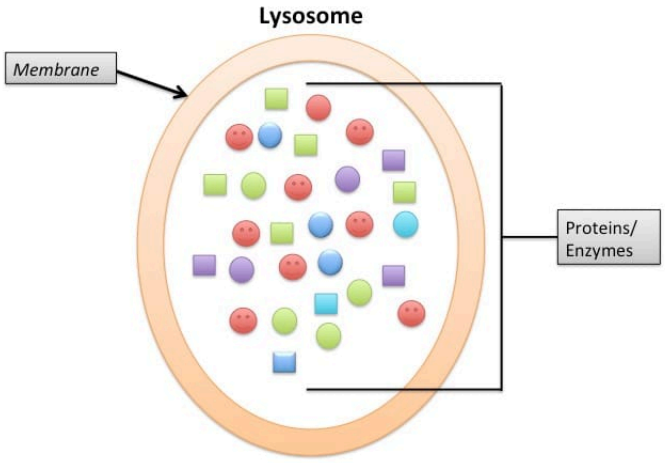


Figure 3.10.1: Structure of Lysosome. of lumoreno (via Wikipedia)

Proteasomes

Proteasomes deal primarily with endogenous proteins; that is, proteins that were synthesized within the cell such as transcription factors, **cyclins** (which must be destroyed to prepare for the next step in the cell cycle) and proteins encoded by viruses and other intracellular pathogens. Proteasomes also address proteins that are folded incorrectly because of translation errors, or they are encoded by faulty genes or they have been damaged by other molecules in the cytosol. Structure of the Proteasome in the Core Particle (CP) and the Regulatory Particle (RP) as shown in Figure 3.10.2.

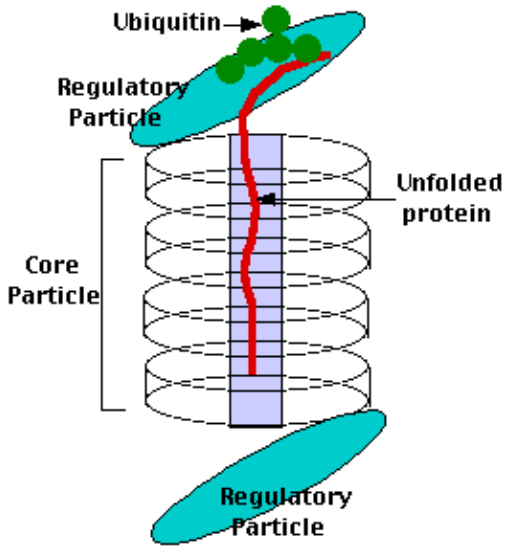


Figure 3.10.2: Simplified scheme of the proteosome complex.

The core particle is made of 2 copies of each of 14 different proteins that are assembled in groups of 7 forming a ring. The 4 rings are stacked on each other (like 4 doughnuts) along a common center (Figure 3.10.3).

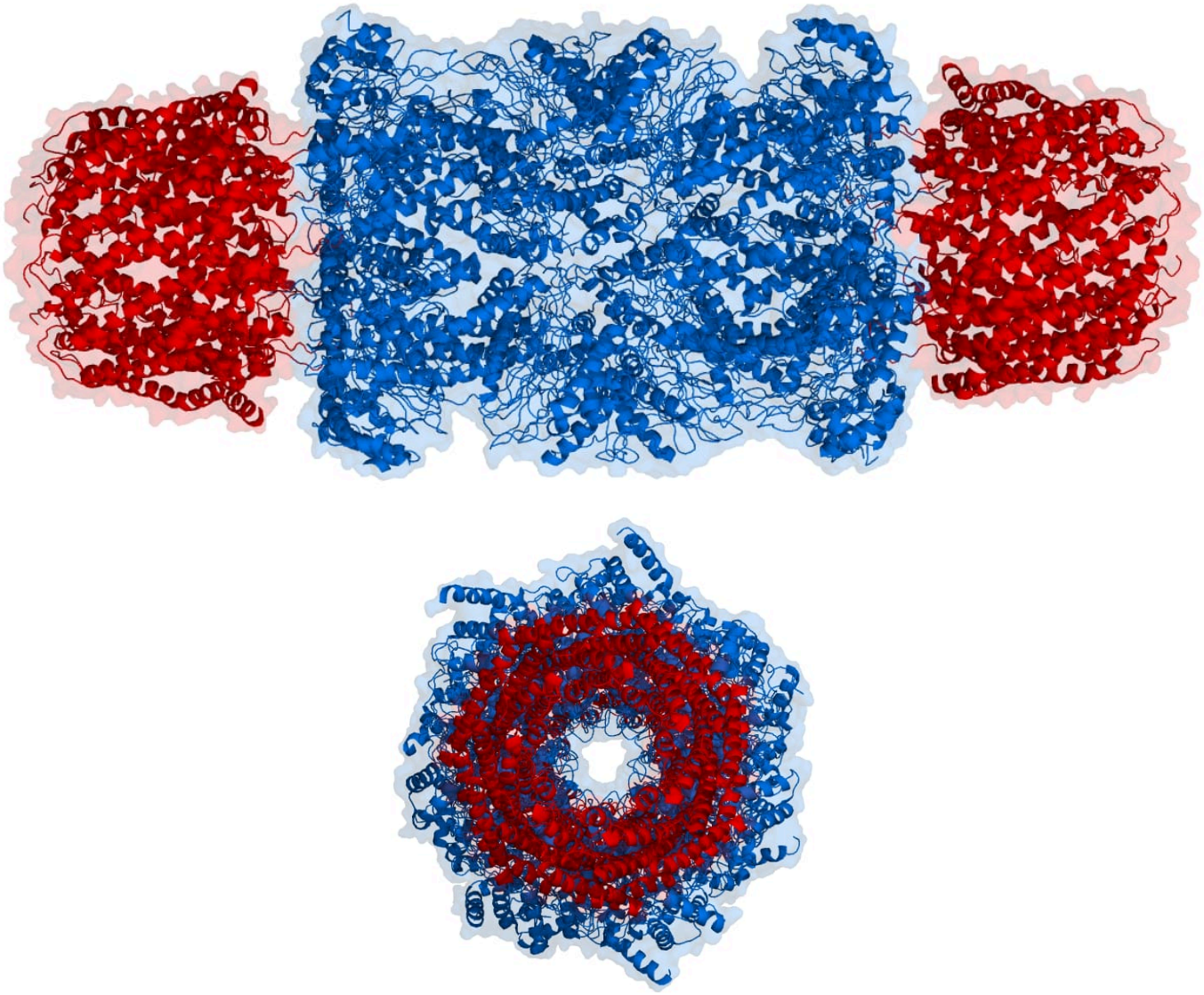


Figure 3.10.3: (left) Cartoon representation of a proteasome. Its active sites are sheltered inside the tube (blue). The caps (red; in this case, 11S regulatory particles) on the ends regulate entry into the destruction chamber, where the protein is degraded. (right) view through the poor. Images used with permission from Wikipedia (CC-SA-BY.3.0; Thomas Splettstoesser).

There are two identical RPs, one at each end of the core particle. Each is made of 19 different proteins (none of them the same as those in the CP). 6 of these are **ATPases** and some of the subunits have sites that recognize the protein **ubiquitin.** Ubiquitin is a small protein (76 amino acids) that is conserved throughout all the kingdoms of life (Figure 3.10.4) and is virtually identical in sequence whether in bacteria, yeast, or mammals. Ubiquitin is used by all these creatures to target proteins for destruction (hence the name based off of the "ubiquitous" term).

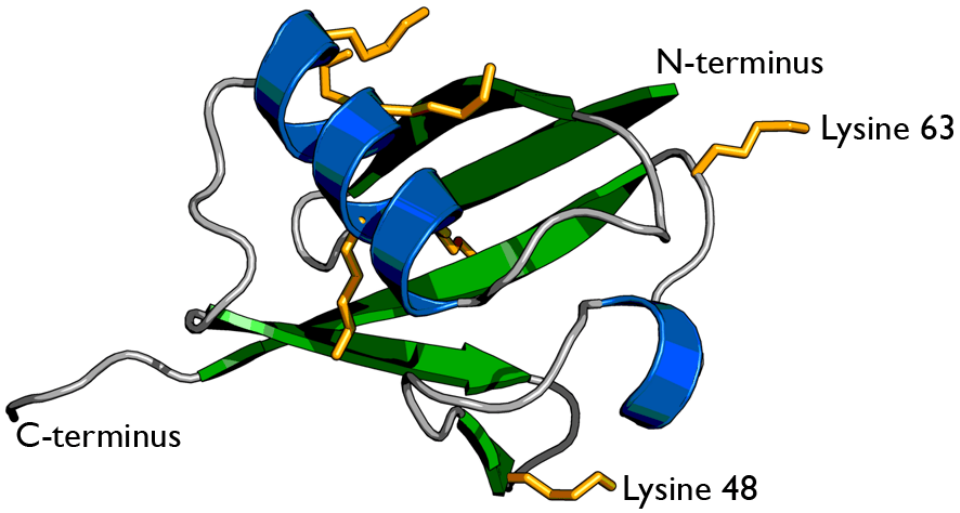


Figure 3.10.4: Cartoon representation of ubiquitin protein, highlighting the secondary structure. from Wikipedia (CC-SA-BY-3.0; Rogerdodd).

The Process

Proteins destined for destruction are conjugated to a molecule of ubiquitin which binds to the terminal amino group of a lysine residue. Additional molecules of ubiquitin bind to the first forming a chain and this complex then binds to ubiquitin-recognizing site(s) on the **regulatory particle.** The protein is unfolded by the ATPases using the energy of ATP, which is translocated into the central cavity of the core particle. Several active sites on the inner surface of the two middle "doughnuts" break various specific peptide bonds of the chain, which produces a set of peptides averaging about 8 amino acids long. These leave the core particle by an unknown route where they may be further broken down into individual amino acids by peptidases in the cytosol. However, in mammals, they may be incorporated in a class I histocompatibility molecule to be presented to the immune system as a potential **antigen.** The regulatory particle releases the ubiquitins for reuse

Antigen Processing by Proteasomes

In **mammals**, activation of the immune system leads to the release of the cytokine **interferon-gamma**. This causes three of the subunits in the core particle to be replaced by substitute subunits; the peptides generated in this altered proteasome are picked up by TAP (= **t**ransporter **a**ssociated with antigen **p**rocessing) proteins and transported from the cytosol into the endoplasmic reticulum where each enters the groove at the surface of a class I histocompatibility molecule. This complex then moves through the Golgi apparatus and is inserted in the plasma membrane where it can be "recognized" by $CD8^+$ **T cells**. It is probably no coincidence that the genes encoding the three substitute **core particle** subunits, **TAP and all the MHC (major histocompatibility complex) molecules** are clustered together on the same chromosome (#6 in humans).

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3.11: The Cytoskeleton

Cells contain elaborate arrays of protein fibers that serve such functions as establishing cell shape, providing mechanical strength, and locomotion. These fibers participate in chromosome separation in mitosis and meiosis and intracellular transport of organelles. The cytoskeleton is made up of three kinds of protein filaments: Actin filaments (also called microfilaments), Intermediate filaments, and Microtubules.

Actin Filaments

Monomers of the protein actin polymerize to form long, thin fibers. These are about 8 nm in diameter and, being the thinnest of the cytoskeletal filaments, are also called **microfilaments** (in skeletal muscle fibers they are called "thin" filaments). Some functions of actin filaments are:

- form a band just beneath the plasma membrane that
  - provides mechanical strength to the cell
  - links transmembrane proteins (e.g., cell surface receptors) to cytoplasmic proteins
  - pinches dividing animal cells apart during cytokinesis
- generate cytoplasmic streaming in some cells
- generate locomotion in cells such as white blood cells and the amoeba
- interact with myosin ("thick") filaments in skeletal muscle fibers to provide the force of muscular contraction

Intermediate Filaments

These cytoplasmic fibers average 10 nm in diameter (and thus are "intermediate" in size between actin filaments (8 nm) and microtubules (25 nm) (as well as of the thick filaments of skeletal muscle fibers). There are several types of intermediate filament, each constructed from one or more proteins characteristic of it.

- **keratins** are found in epithelial cells and also form hair and nails;
- nuclear **lamins** form a meshwork that stabilizes the inner membrane of the nuclear envelope;
- **neurofilaments** strengthen the long axons of neurons;
- **vimentins** provide mechanical strength to muscle (and other) cells.

Despite their chemical diversity, intermediate filaments play similar roles in the cell: providing a supporting framework within the cell. For example, the nucleus in epithelial cells is held within the cell by a basketlike network of intermediate filaments made of **keratins**.

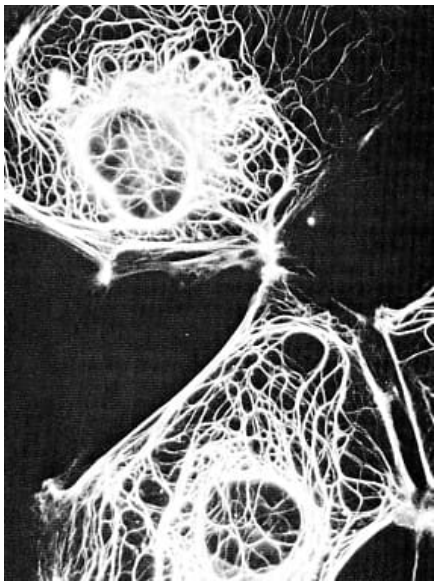


Figure 3.11.1 Intermediate Filaments courtesy Mary Osborn. A fluorescent stain has been used to show the intermediate filaments of keratin in two epithelial cells. Note the basketlike arrangement of filaments around each nucleus.

Different kinds of epithelia use different keratins to build their intermediate filaments. Over 20 different kinds of keratins have been found, although each kind of epithelial cell may use no more than 2 of them. Up to 85% of the dry weight of squamous epithelial cells can consist of keratins.

Microtubules

Microtubules are straight, hollow cylinders whose wall is made up of a ring of 13 "protofilaments" and have a diameter of about 25 nm. They are variable in length but can grow 1000 times as long as they are wide. They are built by the assembly of dimers of **alpha tubulin** and **beta tubulin.** Microtubules are found in both animal and plant cells. In plant cells, microtubules are created at many sites scattered through the cell. In animal cells, the microtubules originate at the **centrosome**. The attached end is called the **minus end**; the other end is the **plus end**.

Microtubules grow at the **plus end** by the polymerization of tubulin dimers (powered by the hydrolysis of GTP), and shrink by the release of tubulin dimers (depolymerization) at the same end. They participate in a wide variety of cell activities. Most involve motion. The motion is provided by protein "motors" that use the energy of ATP to move along the microtubule.

Microtubule motors

There are two major groups of microtubule motors **kinesins** (most of these move toward the plus end of the microtubules) and **dyneins** (which move toward the minus end)

Examples

- The rapid transport of organelles, like vesicles and mitochondria, along the axons of neurons takes place along microtubules with their plus ends pointed toward the end of the axon. The motors are kinesins.
- The migration of chromosomes in mitosis and meiosis takes place on microtubules that make up the **spindle fibers**. Both kinesins and dyneins are used as motors

Cilia and Flagella

Cilia and flagella are built from arrays of microtubules.

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3.12: Cilia

These whiplike appendages extend from the surface of many types of eukaryotic cells. If there are many of them, they are called **cilia**. If only one, or a few, they are **flagella**. Flagella also tend to be longer than cilia but are otherwise similar in construction.

Function of Cilia and Flagella

Cilia and flagella move liquid past the surface of the cell. For single cells, such as sperm, this enables them to **swim**. For cells anchored in a tissue, like the epithelial cells lining our air passages, this moves liquid over the surface of the cell (e.g., driving particle-laden mucus toward the throat). Both cilia and flagella consist of:

- a cylindrical array of 9 filaments consisting of:
  - a complete microtubule (the A-microtubule) extending into the tip of the cilium. When a cilium is being disassembled, protein complexes move down from the tip of the cilium traveling along A-microtubules.
  - a partial microtubule (the B-microtubule) that doesn't extend as far into the tip. When the cilium is growing, its protein components move up toward the tip of the cilium traveling along B-microtubules.
  - cross-bridges of the motor protein **dynein** that extend from the complete microtubule of one filament to the partial microtubule of the adjacent filament.
- a pair of single microtubules running up through the center of the bundle, producing the "9+2" arrangement.
- The entire assembly is sheathed in a membrane that is an extension of the plasma membrane.

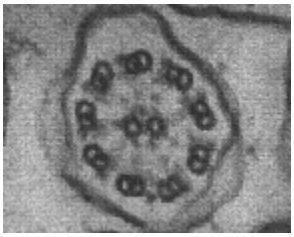


Figure 3.12.1: Cilia courtesy of Peter Satir

This electron micrograph (Figure 3.12.1) shows a cilium in cross section. Each cilium (and flagellum) grows out from, and remains attached to, a **basal body** embedded in the cytoplasm. Basal bodies are identical to centrioles and are, in fact, produced by them. For example, one of the centrioles in developing sperm cells — after it has completed its role in the distribution of chromosomes during meiosis — becomes a basal body and produces the flagellum

The Sliding-Filament Model of Bending

Motion of cilia and flagella is created by the microtubules sliding past one another. This requires motor molecules of **dynein**, which link adjacent microtubules together, and the energy of **ATP**. Dynein powers the sliding of the microtubules against one another — first on one side, then on the other. The bending of cilia (and flagella) has many parallels to the contraction of skeletal muscle fibers.

Testing the Model

Remember, the partial microtubules do not extend as far into the tip as the complete microtubules. So if a slice is made a short distance back from the tip:

- A straight cilium should show the complete pattern (center of diagram).
- In a bent cilium, approximately half the filaments on the upper side should be retracted because of the greater arc on the convex side. So the partial microtubules would disappear being drawn below the plane of the slice. As seen here, bending to the left causes the partial microtubules 4, 5, 6, 7, and 8 to disappear.
- When the cilium bends the other way, the partial microtubules on the opposite side disappear while they reappear on what is now the lower or concave side.

- Electron micrographs (made by Peter Satir) have verified this model precisely.

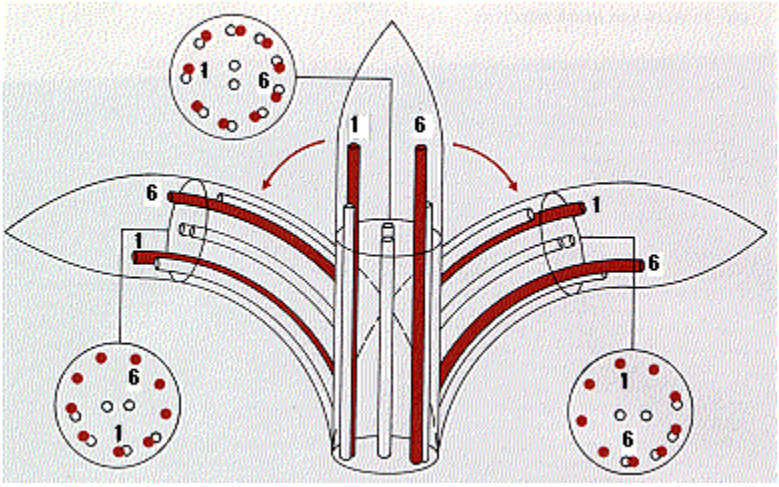


Figure 3.12.1: Cilia Model courtesy Peter Satir

Other Parallels

There are other parallels between the sliding filaments of skeletal muscle and the sliding microtubules of cilia. Both are powered by ATP. Both motors —dynein in cilia, myosin in skeletal muscle — are ATPases and both are regulated by calcium ions.

The Primary Cilium

Motile, "9+2", cilia are found only on certain cells in the vertebrate body, e.g., the epithelia lining the airways. But almost every cell in mammals has — or had — a single **primary cilium**. The primary cilium grows out of the older of the two centrioles that the cell inherited following mitosis. The primary cilium does not beat because it lacks the central pair of microtubules; that is, it is "9+0". Where functions have been identified, they all involve sensory reception. Some examples are as follows:

- **Mechanoreceptors**: A primary cilium extends from the apical surface of the epithelial cells lining the kidney tubules and monitors the flow of fluid through the tubules. Inherited defects in the formation of these cilia cause polycystic kidney disease.
- **Chemoreceptors**: We detect odors by receptors on the primary cilium of olfactory neurons. Many types of cells detect extracellular signaling molecules, e.g., nutrients, growth factors, hormones, with receptors localized on their primary cilium. These signals may be transduced into the nucleus where they alter gene expression.
- **Photoreceptors**: The outer segment of the rods in the vertebrate retina is also derived from a primary cilium.

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3.13: Animal Tissues

The development of a fertilized egg into a newborn child requires an average of 41 rounds of mitosis ($2^{41} = 2.2 \times 10^{12}$). During this period, the cells produced by mitosis enter different pathways of differentiation; some becoming blood cells, some muscle cells, and so on. There are more than 100 visibly-distinguishable kinds of differentiated cells in the vertebrate animal. These are organized into tissues; the tissues into organs. Groups of organs make up the various systems — digestive, excretory, etc. — of the body (Figure 3.13.1 and Table 3.13.1).

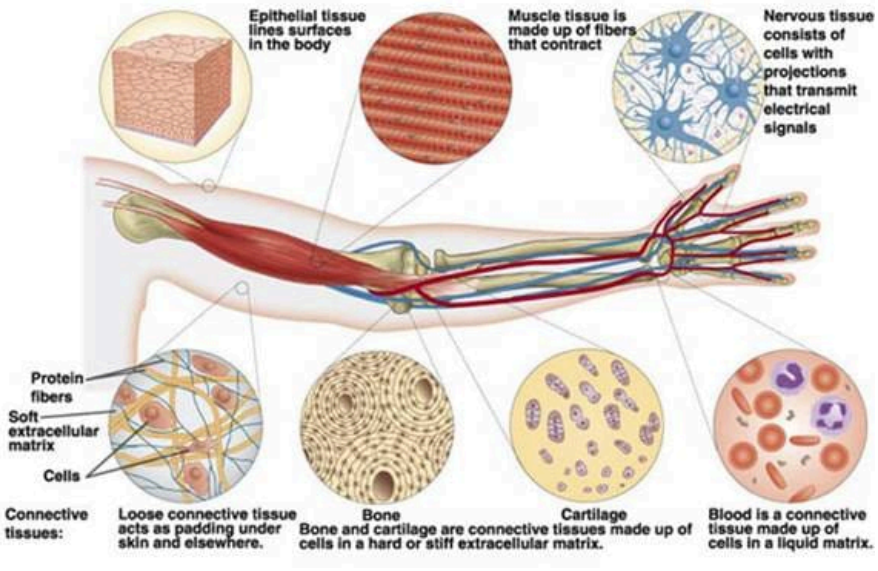


Figure 3.13.1: Animal Tissues

The actual number of differentiated cell types is surely much larger than 100. All lymphocytes, for example, look alike but actually represent a variety of different functional types, e.g., B cells, T cells of various subsets. The neurons of the central nervous system must exist in a thousand or more different functional types, each representing the result of a particular pathway of differentiation. This page will give a brief introduction to the major types of animal tissues.

Table 3.13.1: Classification of Animal Tissues

Epithelial Tissues	Linings and Coverings	Simple Epithelia
	Classifying or Naming Epithelia	Stratified Epithelia
	Glands	Exocrine Glands
		Endocrine Glands
Connective Tissues	Fluid Connective Tissues	Lymph
		Blood
	Connective Tissues Proper	Loose Connective Tissues
		Loose Connective Tissues and Inflammation
		Dense Connective Tissues
	Supportive Connective Tissues	Osseous Tissue
		Cartilage
Muscle Tissues	Non-striated	Smooth muscle
	Striated	Skeletal Muscle

		Cardiac Muscle
Nervous Tissues	Neurons	Multipolar Neurons in CNS
	Nerves	Nerves of the PNS
	Receptors	Miessner's and Pacinian Corpuscles

Epithelial

Epithelial tissue is made of closely-packed cells arranged in flat sheets. Epithelia form the surface of the skin, line the various cavities and tubes of the body, and cover the internal organs. Epithelia that form the interface between the internal and external environments. Skin as well as the lining of the mouth and nasal cavity. These are derived from **ectoderm**. Inner lining of the GI tract, lungs, urinary bladder, exocrine glands, vagina and more. These are derived from **endoderm**.

The **apical surface** of these epithelial cells is exposed to the "external environment", the lumen of the organ or the air.

- **Mesothelia**. These are derived from mesoderm.
  - pleura — the outer covering of the lungs and the inner lining of the thoracic (chest) cavity.
  - peritoneum — the outer covering of all the abdominal organs and the inner lining of the abdominal cavity.
  - pericardium — the outer lining of the heart.
- **Endothelia**. These are derived from mesoderm. The inner lining of the heart, all blood and lymphatic vessels.

The **basolateral surface** of all epithelia is exposed to the internal environment - extracellular fluid (ECF). The entire sheet of epithelial cells is attached to a layer of extracellular matrix that is called the basement membrane or, better (because it is not a membrane in the biological sense), the **basal lamina**.

The function of epithelia always reflects the fact that they are boundaries between masses of cells and a cavity or space. Some examples include:

- The epithelium of the skin protects the underlying tissues from mechanical damage, ultraviolet light, dehydration and invasion by bacteria
- The columnar epithelium of the intestine secretes digestive enzymes into the intestine and absorbs the products of digestion from it.
- An epithelium also lines our air passages and the alveoli of the lungs. It secretes mucus which keeps it from drying out and traps inhaled dust particles. Most of its cells have cilia on their apical surface that propel the mucus with its load of foreign matter back up to the throat.

Muscle

Three kinds of muscle are found in vertebrates. **Skeletal muscle** is made of long fibers whose contraction provides the force of locomotion and other **voluntary** body movements. **Smooth muscle** lines the walls of the hollow structures of the body, such as the intestine, urinary bladder, uterus, and blood vessels. Its contraction, which is **involuntary**, reduces the size of these hollow organs. The heart is made of **cardiac muscle**.

Connective

The cells of connective tissue are embedded in a great amount of extracellular material. This **matrix** is secreted by the cells. It consists of protein fibers embedded in an amorphous mixture of protein-polysaccharide ("proteoglycan") molecules. Supporting connective tissue gives strength, support, and protection to the soft parts of the body.

- **cartilage**. Example: the outer ear
- **bone**. The matrix of bone contains collagen fibers and mineral deposits. The most abundant mineral is calcium phosphate, although magnesium, carbonate, and fluoride ions are also present.

Dense connective tissue is often called fibrous connective tissue and include **Tendons** and **Ligaments**. Tendons connect muscle to bone with a The matrix is principally Type I collagen, and the fibers are all oriented parallel to each other. Tendons are strong

but not elastic. **Ligaments** attach one bone to another and contain both collagen and also the protein **elastin**. Elastin permits ligaments to be stretched.

Loose connective tissue is distributed throughout the body. It serves as a packing and binding material for most of our organs. Sheets of loose connective tissue that bind muscles and other structures together are called **fascia**. Collagen, elastin, and other proteins are found in the matrix of loose connective tissue. Both dense and loose connective tissue are derived from cells called **fibroblasts**, which secrete the extracellular matrix.

Adipose Tissue

Adipose tissue is "fat". There are two kinds found in mammals: **white adipose tissue** (WAT) and **brown adipose tissue** (BAT). The WAT in which the cells, called **adipocytes**, have become almost filled with oil, which is confined within a single membrane-enclosed droplet. Virtually all of the "fat" in adult humans is white adipose tissue. BAT in which the adipocytes contain many small droplets of oil as well as many mitochondria. White adipose tissue and brown adipose tissue differ in function as well as cellular structure. These differences are described elsehwhere.

New adipocytes in white adipose tissue are formed throughout life from a pool of precursor cells. These are needed to replace those that die (after an average life span of 10 years). Whether the total **number** of these adipocytes increases in humans becoming fatter as adults is still uncertain. If not, why do so many of us get fatter as we age? Because of the increased **size** of individual adipocytes as they become filled with oil. The adipocytes of white adipose tissue secrete several hormones, including leptin and adiponectin.

Nerve

Nerve tissue is composed of nerve cells called neurons and glial cells. Neurons are specialized for the conduction of nerve impulses; a typical neuron consists of a **cell body** which contains the nucleus; a number of short fibers — **dendrites** — extending from the cell body and a single long fiber, the **axon**. The nerve impulse is conducted along the axon. The tips of axons meet other neurons at junctions called **synapses, muscles (called neuromuscular junctions) and** glands.

Glia

Glial cells surround neurons. Once thought to be simply support for neurons (*glia* = glue), they turn out to serve several important functions. There are three types:

- **Schwann cells**. These produce the myelin sheath that surrounds many axons in the peripheral nervous system.
- **Oligodendrocytes**. These produce the myelin sheath that surrounds many axons in the central nervous system (brain and spinal cord).
- **Astrocytes**. These, often star-shaped cells are clustered around synapses and the nodes of Ranvier where they perform a variety of functions such as:
  - modulating the activity of neurons
  - supplying neurons with materials (e.g. glucose and lactate) as well as some signaling molecules
  - regulating the flow of blood to their region of the brain. It is primarily the metabolic activity of astrocytes that is being measured in brain imaging by positron-emission tomography (PET) and functional magnetic resonance imaging (fMRI).
  - pruning away (by phagocytosis) weak synapses

In addition, the central nervous system contains many **microglia** — mobile cells (macrophages) that respond to damage (e.g., from an infection) by engulfing cell debris and secreting inflammatory cytokines like tumor necrosis factor (TNF-α) and interleukin-1 (IL-1). Microglia are also active in the healthy brain, at least in young mice where, like astrocytes, they engulf synapses thus reducing the number of synapses in the developing brain.

Blood

The **bone marrow** is the source of all the cells of the blood. These include **red blood cells** (RBCs or **erythrocytes**), five kinds of **white blood cells** (WBCs or **leukocytes**), and **platelets** (or **thrombocytes**).

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3.14: Adipose Tissue

Two kinds of adipose tissue are found in mammals: white adipose tissue (WAT) and brown adipose tissue (BAT). White adipose tissue is the most common and is the fat that so many of us complain of acquiring. Brown adipose tissue is present in small mammals (e.g., mice) and in newborn humans. Most of it disappears in adult humans.The cells in both types of fat are called adipocytes although they differ in origin, structure, and function in the two types of tissue.

Table 3.14.1: Two classifications of Adipocytes

WAT Adipocytes	BAT Adipocytes
a narrow rim of cytoplasm with its nucleus pressed near the margin of the cell surrounding	Cytoplasm throughout the cell with a central nucleus and
a single large membrane-enclosed lipid droplet	many small lipid droplets
few mitochondria	many mitochondria (providing the brown color)
modest blood supply	rich blood supply
serves as a depot of stored energy	function is to generate heat

New adipocytes in **white** adipose tissue are formed throughout life from a pool of precursor cells. These are needed to replace those that die (after an average life span of 10 years). Whether the total **number** of these adipocytes increases in humans becoming fatter as adults is still uncertain. If not, why do so many of us get fatter as we age? Because of the increased **size** of individual adipocytes as they become filled with oil.

The adipocytes of white adipose tissue secrete several hormones, including leptin, asprosin (which, during fasting, causes a rapid release of blood sugar (glucose) from the liver) and adiponectin. In addition to serving as a major source of energy reserves, white adipose tissue also provides some mechanical protection and insulation to the body. Obesity is the excessive accumulation of white adipose tissue.

Brown adipose tissue provides a vital source of heat to maintain body temperature in small mammals (with their high surface to volume ratio) and infants (who usually cannot shiver when they are cold).

Adipose tissue activation mechanism

Brown adipose tissue is activated by the following mechanism when the body temperature drops:

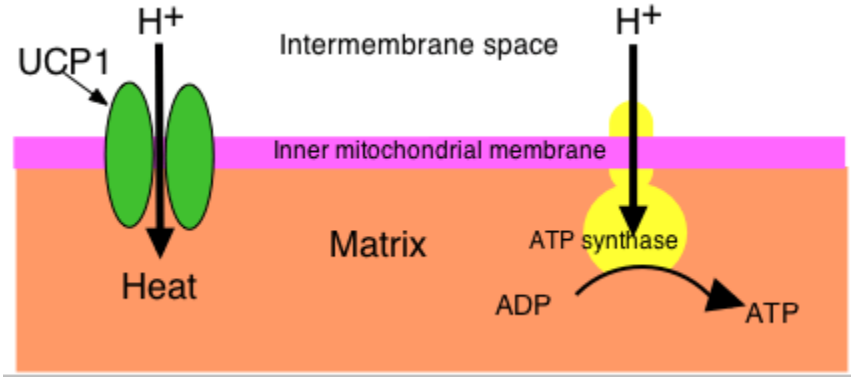


Figure 3.14.1 Adipose tissue activation process

- Cold activates the sympathetic nervous system.
- Noradrenaline is released by the postganglionic neurons.
- The noradrenaline binds to G-protein-coupled receptors on the adipocytes surface.

- The second messenger cAMP is generated and moves into the nucleus where
- it binds to the promoter of the gene encoding an enzyme that converts thyroxine (T4) to triiodothyronine (T3).
- T3 enters the nucleus and bind to the promoter of the gene encoding uncoupling protein1 (**UCP1**).
- UCP1 inserts into the inner membrane of the mitochondria where
- it allows the protons that have been pumped out into the intermembrane space by the electron transport chain
- to return to the matrix without having to pass through ATP synthase.

So instead of cellular respiration (of fatty acids and glucose) generating ATP, it generates heat.

WAT can Acquire the Properties of BAT

In mice and perhaps in humans, skeletal muscles that have undergone a period of vigorous exercise secrete a protein hormone called **irisin**. Irisin acts on white adipose tissue to give it the properties of brown adipose tissue:

- an increase in the number of mitochondria and lipid droplets;
- a marked increase in the synthesis of **UCP1**;
- an increase in the rate of cellular respiration but with the energy released as heat rather than fueling the synthesis of ATP.

These brown-like fat cells derived from white fat cells have been called "beige" or "brite" cells. Lean adult humans have deposits of beige cells in the neck and upper chest regions. When they are exposed to cold, their beige cells are activated. Obese people have few or no beige cells. Probably their layers of white adipose tissue provide such good insulation that they are in less danger of heat loss.

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3.15: Junctions between Cells

In many animal tissues (e.g., connective tissue), each cell is separated from the next by an extracellular coating or matrix. However, in some tissues (e.g., epithelia), the plasma membranes of adjacent cells are pressed together. Four kinds of junctions occur in vertebrates:

1. Tight junctions
2. Adherens junctions
3. Gap junctions
4. Desmosomes

In many plant tissues, it turns out that the plasma membrane of each cell is continuous with that of the adjacent cells. The membranes contact each other through openings in the cell wall called Plasmodesmata.

Tight Junctions

Epithelia are sheets of cells that provide the interface between masses of cells and a cavity or space (a lumen). The portion of the cell exposed to the lumen is called its **apical** surface. The rest of the cell (i.e., its sides and base) make up the **basolateral** surface. Tight junctions seal adjacent epithelial cells in a narrow band just beneath their apical surface. They consist of a network of **claudins** and other proteins. Tight junctions perform two vital functions:

1. They limit the passage of molecules and ions through the space between cells. So most materials must actually enter the cells (by diffusion or active transport) in order to pass through the tissue. This pathway provides tighter control over what substances are allowed through.
2. They block the movement of integral membrane proteins (red and green ovals) between the apical and basolateral surfaces of the cell. Thus the special functions of each surface, for example receptor-mediated endocytosis at the apical surface and exocytosis at the basolateral surface can be preserved.

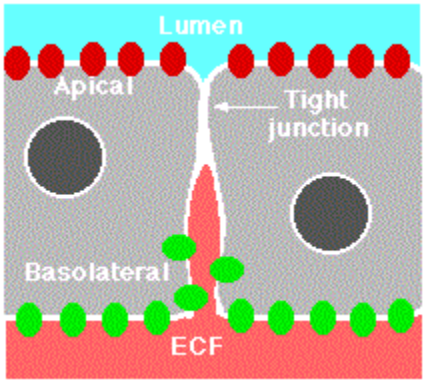


Figure 3.15.1: Tight Junction

The Epithelia of the Human Lung

A report by Vermeer, et al., in the 20 March 2003 issue of Nature provides a striking example of the role of tight junctions. The epithelial cells of the human lung express

- a growth stimulant, called **heregulin**, on their **apical** surface and
- its receptors on the **basolateral** surface. (These receptors also respond to epidermal growth factor (**EGF**), and mutant versions have been implicated in cancer.

As long as the sheet of cells is intact, there is no stimulation of its receptors by heregulin thanks to the seal provided by tight junctions. However, if the sheet of cells becomes broken, heregulin can reach its receptors. The result is an autocrine stimulation of mitosis leading to healing of the wound. Several disorders of the lung the chronic bronchitis of cigarette smokers, asthma, cystic fibrosis increase the permeability of the airway epithelium. The resulting opportunity for autocrine stimulation may account for the proliferation (piling up) of the epithelial cells characteristic of these disorders.

Adherens Junctions

Adherens junctions provide strong mechanical attachments between adjacent cells. They hold **cardiac muscle** cells tightly together as the heart expands and contracts and hold **epithelial cells** together. Adherens junctions seem to be responsible for contact inhibition and some are present in narrow bands connecting adjacent cells. Others are present in discrete patches holding the cells together . Adherens junctions are built from **cadherins** that are transmembrane proteins (Figure 3.15.2; red labeled) whose extracellular segments bind to each other and whose intracellular segments bind to **catenins** (Figure 3.15.2; yellow labeled), which are connected to actin filaments

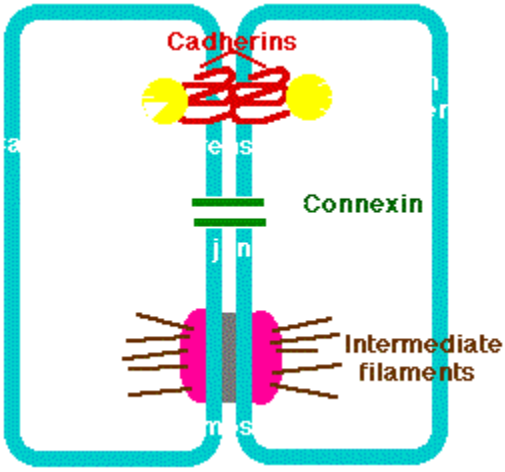


Figure 3.15.2: Adheren Junctions

Human synthesize some 80 different types of cadherins. In most cases, a cell expressing one type of cadherin will only form adherens junctions with another cell expressing the same type. This is because molecules of cadherin tend to form homodimers, not heterodimers. Inherited mutations in a gene encoding a cadherin can cause stomach cancer. Mutations in a gene (*APC*), whose protein normally interacts with catenins, are a common cause of colon cancer. Loss of functioning adherens junctions may accelerate the edema associated with sepsis and tumor metastasis.

Gap Junctions

Gap junctions are intercellular channels some 1.5–2 nm in diameter. These permit the free passage between the cells of ions and small molecules (up to a molecular weight of about 1000 daltons). They are cylinders constructed from 6 copies of transmembrane proteins called **connexins**. Because ions can flow through them, gap junctions permit changes in membrane potential to pass from cell to cell.

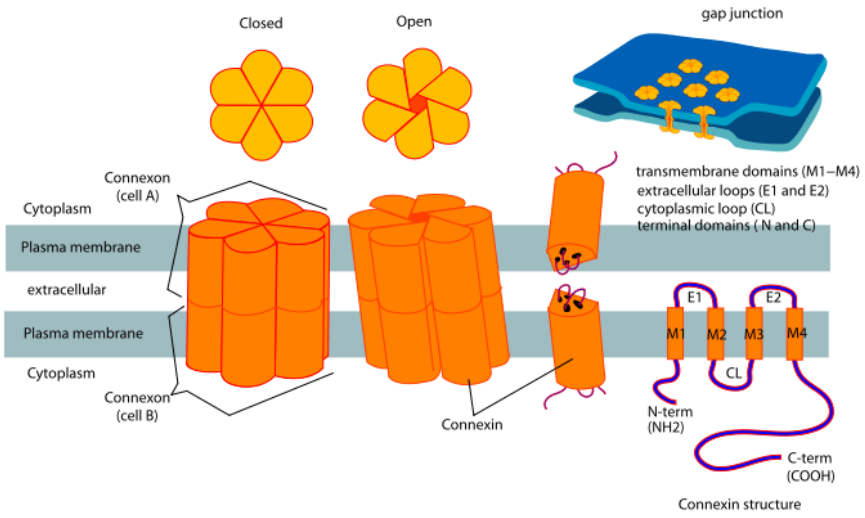


Figure 3.15.3: The diagram shows a gap junction and its main element. connexon. together with the structure of the connexin. (CC-BY-SA-3.0; Mariana Ruiz)

Examples of gap junctions include:

- The action potential in heart (cardiac) muscle flows from cell to cell through the heart providing the rhythmic contraction of the heartbeat.
- At some so-called electrical synapses in the brain, gap junctions permit the arrival of an action potential at the synaptic terminals to be transmitted across to the postsynaptic cell without the delay needed for release of a neurotransmitter.
- As the time of birth approaches, gap junctions between the smooth muscle cells of the uterus enable coordinated, powerful contractions to begin.

Several inherited disorders of humans such as certain congenital heart defects and certain cases of congenital deafness have been found to be caused by mutant genes encoding connexins.

Desmosomes

Desmosomes are localized patches that hold two cells tightly together. They are common in epithelia (e.g., the skin). Desmosomes are attached to intermediate filaments of keratin in the cytoplasm. **Pemphigus** is an autoimmune disease in which the patient has developed antibodies against proteins (cadherins) in desmosomes. The loosening of the adhesion between adjacent epithelial cells causes blistering. Carcinomas are cancers of epithelia. However, the cells of carcinomas no longer have desmosomes. This may partially account for their ability to metastasize.

Hemidesmosomes

These are similar to desmosomes but attach epithelial cells to the basal lamina ("basement membrane") instead of to each other. **Pemphigoid** is an autoimmune disease in which the patient develops antibodies against proteins (integrins) in hemidesmosomes. This, too, causes severe blistering of epithelia.

Plasmodesmata

Although each plant cell is encased in a boxlike cell wall, it turns out that communication between cells is just as easy, if not easier, than between animal cells (Figure 3.15.4). Fine strands of cytoplasm, called **plasmodesmata**, extend through pores in the cell wall connecting the cytoplasm of each cell with that of its neighbors.

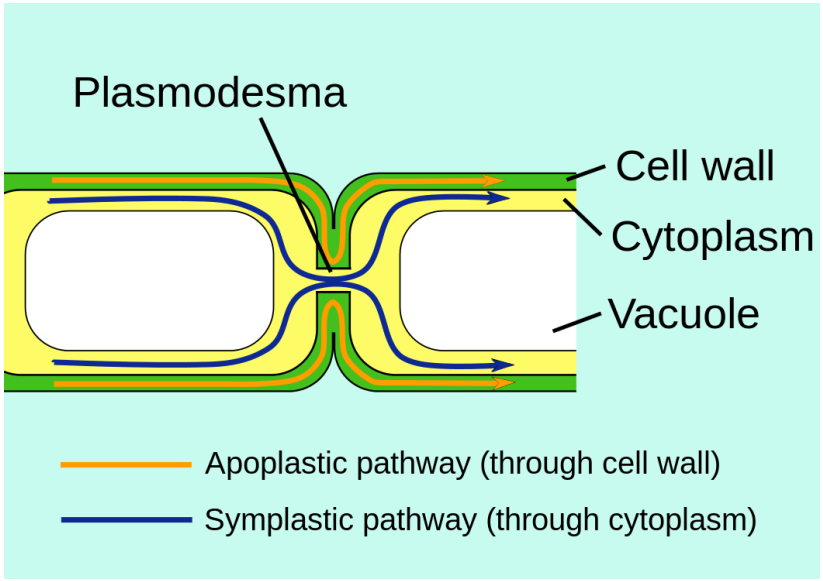


Figure 3.15.4: A diagram showing the different routes through which water can travel through a plant. Plasmodesmata allow molecules to travel between plant cells through the symplastic pathway. Image used wtih permission from Wikipedia (public domain; Jackacon).

Plasmodesmata provide an easy route for the movement of ions, small molecules like sugars and amino acids, and even macromolecules like RNA and proteins, between cells. The larger molecules pass through with the aid of actin filaments. Plasmodesmata are sheathed by a plasma membrane that is simply an extension of the plasma membrane of the adjoining cells. This raises the intriguing question of whether a plant tissue is really made up of separate cells or is, instead, a syncytium: a single, multinucleated cell distributed throughout hundreds of tiny compartments.

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3.16: Plant Cells

Plant cells are eukaryotic and have many of the structures found in animal cells.

- Plasma membrane
- Nucleus and nucleolus
- Mitochondria
- Ribosomes
- Endoplasmic reticulum
- Golgi apparatus
- Peroxisomes (the crystal in the electron micrograph is enclosed within a peroxisome)
- Microtubules

Plant cells differ from animal cells as they lack centioles and intermediate filament; they also do not have plastids and a cell wall and large vacuoles.

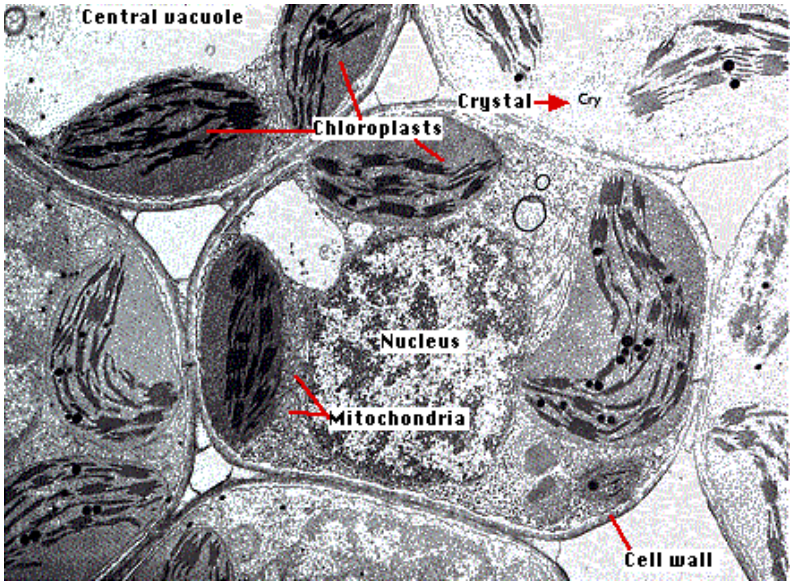


Figure 3.16.1 Sunflower leaf micrograph courtesy of H. J. Arnott and Kenneth M. Smith

Plastids

Chloroplasts are the most familiar plastids. They are usually disk-shaped and about 5-8 µm in diameter and 2-4 µm thick. A typical plant cell has 20-40 of them. Chloroplasts are green because they contain chlorophylls — the pigments that harvest the light used in photosynthesis. Chloroplasts are probably the descendants of cyanobacteria that took up residence in the ancestor of the plants. Plant cells that are not engaged in photosynthesis also have plastids that serve other functions such as storing starch (when they are called **leucoplasts**) and storing the carotenoids that give flowers and fruits their color (when they are called **chromoplasts**)

The Cell Wall

The rigid cell wall of plants is made of fibrils of cellulose embedded in a matrix of several other kinds of polymers such as pectin and lignin. The linear nature of cellulose molecules and the many opportunities for side-to-side intermolecular hydrogen bonding provide just what one would want to build long and stiff fibrils.

- **Primary cell walls:** The cell walls of parenchyma and meristems are uniform in thickness and are primary cell walls. Although each cell appears encased within a box, in fact primary cell walls are perforated permitting plasmodesmata to connect

adjacent cells.

- **Secondary cell walls:** The cells of sclerenchyma, collenchyma and xylem have secondary deposits of lignified cellulose which provide mechanical strength to the tissue.

Vacuoles

Vacuoles are enclosed by a single membrane. Young plant cells often contain many small vacuoles, but as the cells mature, these unite to form a large **central vacuole**. Vacuoles serve several functions such as:

- storing foods (e.g., proteins in seeds)
- storing wastes
- storing malic acid in CAM plants
- storing various ions (e.g., calcium, sodium, iron) which, among other functions, helps to
- maintain turgor in the cell.

Plant cells avoid bursting in hypotonic surroundings by their strong cell walls. These allow the build-up of **turgor** within the cell. Loss of turgor causes wilting.

Plasmolysis

When a freshwater (or terrestrial) plant is placed in sea water, its cells quickly lose turgor and the plant wilts. This is because sea water is hypertonic to the cytoplasm. As water diffuses from the cytoplasm into the sea water, the cells shrink — drawing their plasma membrane away from the cell wall.

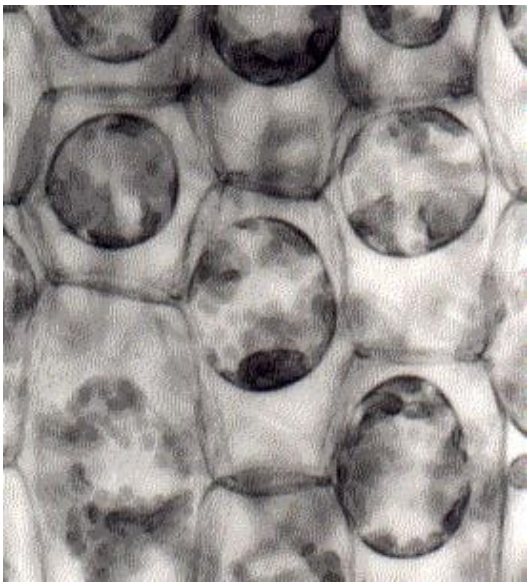


Figure 3.16.2: Plasmolyzed cell of freshwater plant Elodea

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3.17: Chloroplasts

A typical plant cell (e.g., in the palisade layer of a leaf) might contain as many as 50 chloroplasts.

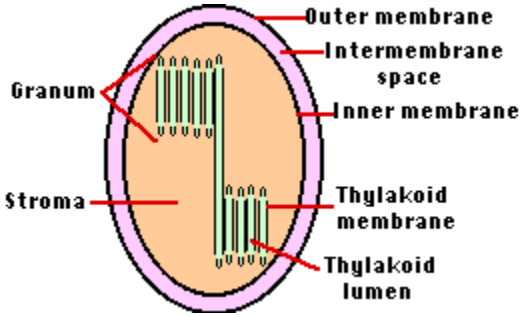


Figure 3.17.1 Typical Chloroplast

The chloroplast is made up of 3 types of membrane:

- A smooth **outer membrane** which is freely permeable to molecules.
- A smooth **inner membrane** which contains many **transporters**: integral membrane proteins that regulate the passage in an out of the chloroplast of
  - small molecules like sugars
  - proteins synthesized in the cytoplasm of the cell but used within the chloroplast
- A system of **thylakoid membranes**

Thylakoids

The thylakoid membranes enclose a lumen: a system of vesicles (that may all be interconnected). At various places within the chloroplast these are stacked in arrays called **grana** (resembling a stack of coins). Four types of protein assemblies are embedded in the thylakoid membranes: These carry out the so-called light reactions of photosynthesis including:

1. **Photosystem I** which includes chlorophyll and carotenoid molecules
2. **Photosystem II** which also contains chlorophyll and carotenoid molecules
3. **Cytochromes b** and **f**
4. **ATP synthase**

The thylakoid membranes are surrounded by a fluid **stroma,** which contains all the enzymes, e.g., RUBISCO, needed to carry out the "dark" reactions of photosynthesis; that is, the conversion of CO_2 into organic molecules like glucose. A number of identical molecules of DNA, each of which carries the complete chloroplast genome. The genes encode some — but not all of the molecules needed for chloroplast function. The others are

- transcribed from genes in the **nucleus** of the cell
- translated in the cytoplasm and
- transported into the chloroplast.

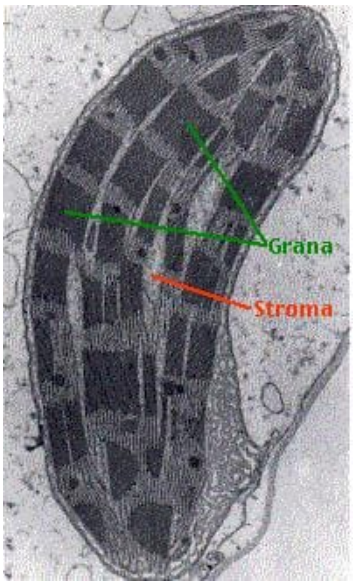


Figure 3.17.2 Chloroplast from a corn cell courtesy of Dr. L. K. Shumway

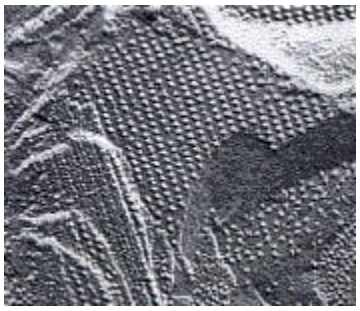


Figure 3.17.3 Inner surface of thylakoid courtesy of Kenneth R. Miller

The electron micrograph in Figure 3.17.3 shows the inner surface of a thylakoid membrane. Each particle may represent one photosystem II complex. In the functioning chloroplast, these particles may not be as highly ordered as seen here.

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3.18: Chlorophylls and Carotenoids

Chlorophylls

Two types of chlorophyll are found in plants and the green algae: **chlorophyll a** and **chlorophyll b.** The difference in their structures is shown in the above figure (red disks).

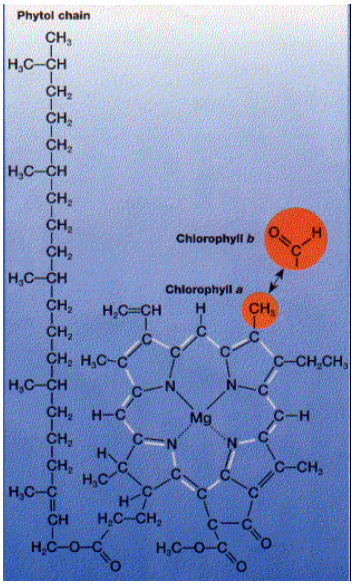


Figure 3.18.1 Chlorophyll

In the chloroplast, both types are associated with integral membrane proteins in the thylakoid membrane. Note the system of alternating single and double bonds (white bars) that run around the porphyrin ring. Although I am forced to draw the single and double bonds in fixed positions, actually the "extra" electrons responsible for the double bonds are not fixed between any particular pair of carbon atoms but instead are free to migrate around the ring. This property enables these molecules to absorb light. Both chlorophylls absorb light most strongly in the red and violet parts of the spectrum. Green light is absorbed poorly. Thus when white light shines on chlorophyll-containing structures like leaves, green light is transmitted and reflected and the structures appear green.

Carotenoids

Chloroplasts also contain carotenoids. These are also pigments with colors ranging from red to yellow. Carotenoids absorb light most strongly in the blue portion of the spectrum. They thus enable the chloroplast to trap a larger fraction of the radiant energy falling on it. Carotenoids are often the major pigments in **flowers** and **fruits**. The red of a ripe tomato and the orange of a carrot are produced by their carotenoids. In leaves, the carotenoids are usually masked by the chlorophylls. In the autumn, as the quantity of chlorophyll in the leaf declines, the carotenoids become visible and produce the yellows and reds of autumn foliage.

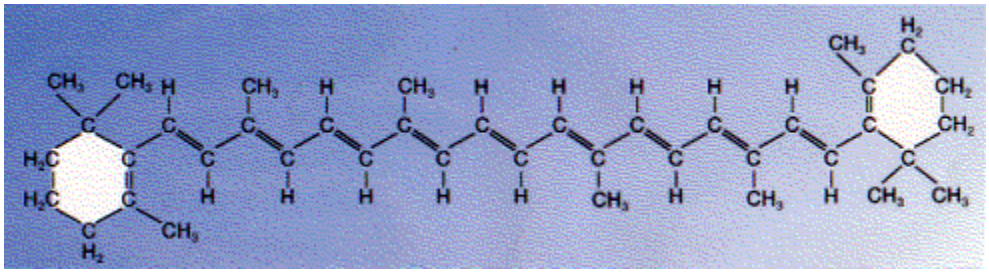


Figure 3.18.2 Carotenoids

Figure 3.18.2 shows the structure of **beta-carotene**, one of the most abundant carotenoids. Note again the system of alternating single and double bonds that in this molecule runs along the hydrocarbon chain that connects the two benzene rings. As in chlorophyll, the electrons of the double bonds actually migrate though the chain and also make this molecule an efficient absorber of light. Many animals use ingested beta-carotene as a precursor for the synthesis of vitamin A.

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3.19: Plant Tissues

A mature **vascular plant** (any plant other than mosses and liverworts), contains several types of differentiated cells. These are grouped together in tissues. Some tissues contain only one type of cell. Others consist of several cells.

Meristematic

The main function of meristematic tissue is mitosis. The cells are small, thin-walled, with no central vacuole and no specialized features. Meristematic tissue is located in the **apical meristems** at the growing points of roots and stems, the **secondary meristems** (lateral buds) at the **nodes** of stems (where branching occurs), and meristematic tissue, called the **cambium**, that is found within mature stems and roots. The cells produced in the meristems soon become differentiated into one or another of several types.

Protective

Protective tissue covers the surface of leaves and the living cells of roots and stems. Its cells are flattened with their top and bottom surfaces parallel. The upper and lower **epidermis of the leaf** are examples of protective tissue.

Parenchyma

The cells of parenchyma are large, thin-walled, and usually have a large central vacuole. They are often partially separated from each other and are usually stuffed with plastids. In areas not exposed to light, colorless plastids predominate and food storage is the main function. The cells of the white potato are parenchyma cells. Where light is present, e.g., in leaves, chloroplasts predominate and photosynthesis is the main function.

Sclerenchyma

The walls of these cells are very thick and built up in a uniform layer around the entire margin of the cell. Often, the cell dies after its cell wall is fully formed. Sclerenchyma cells are usually found associated with other cells types and give them mechanical support. Sclerenchyma is found in stems and also in leaf veins. Sclerenchyma also makes up the hard outer covering of seeds and nuts.

Collenchyma

Collenchyma cells have thick walls that are especially thick at their corners. These cells provide mechanical support for the plant. They are most often found in areas that are growing rapidly and need to be strengthened. The **petiole** ("stalk") of leaves is usually reinforced with collenchyma.

Xylem

Xylem conducts water and dissolved minerals from the roots to all the other parts of the plant. In angiosperms, most of the water travels in the **xylem vessels**. These are thick-walled tubes that can extend vertically through several feet of xylem tissue. Their diameter may be as large as 0.7 mm. Their walls are thickened with secondary deposits of cellulose and are usually further strengthened by impregnation with **lignin**. The secondary walls of the xylem vessels are deposited in spirals and rings and are usually perforated by pits.

Xylem vessels arise from individual cylindrical cells oriented end to end. At maturity the end walls of these cells dissolve away, and the cytoplasmic contents die. The result is the xylem vessel, a continuous nonliving duct. Xylem also contains **tracheids**. These are individual cells tapered at each end so the tapered end of one cell overlaps that of the adjacent cell. Like xylem vessels, they have thick, lignified walls and, at maturity, no cytoplasm. Their walls are perforated so that water can flow from one tracheid to the next. The xylem of ferns and conifers contains only tracheids. In woody plants, the older xylem ceases to participate in water

transport and simply serves to give strength to the trunk. Wood is xylem. When counting the annual rings of a tree, one is counting rings of xylem.

Phloem

The main components of phloem are **sieve elements** and **companion cells**. **Sieve elements** are so-named because their end walls are perforated. This allows cytoplasmic connections between vertically-stacked cells. The result is a **sieve tube** that conducts the products of photosynthesis — sugars and amino acids — from the place where they are manufactured (a "source"), e.g., leaves, to the places ("sinks") where they are consumed or stored; such as

- roots
- growing tips of stems and leaves
- flowers
- fruits, tubers, corms, etc.

Sieve elements have no nucleus and only a sparse collection of other organelles. They depend on the adjacent companion cells for many functions.

Companion cells move sugars, amino acids and a variety of macromolecules into and out of the sieve elements. In "source" tissue, such as a leaf, the companion cells use transmembrane proteins to take up — by active transport — sugars and other organic molecules from the cells manufacturing them. Water follows by osmosis. These materials then move into adjacent sieve elements through plasmodesmata. The pressure created by osmosis drives the flow of materials through the sieve tubes.

In "sink" tissue, the sugars and other organic molecules leave the sieve elements through plasmodesmata connecting the sieve elements to their companion cells and then pass on to the cells of their destination. Again, water follows by osmosis where it may

- leave the plant by transpiration or
- increase the volume of the cells or
- move into the xylem for recycling through the plant

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3.20: Apoptosis

Apoptosis is a process of programmed cell death that occurs in multicellular organisms. There are two ways in which cells die: (1) They are killed by injurious agents or (2) they are induced to commit suicide.

Death by injury

Cells that are damaged by injury, such as by mechanical damage or exposure to toxic chemicals undergo a characteristic series of changes. They (and their organelles like mitochondria) swell (because the ability of the plasma membrane to control the passage of ions and water is disrupted). The cell contents leak out, leading to inflammation of surrounding tissues.

Death by Suicide

Cells that are induced to commit suicide:

- shrink
- develop bubble-like blebs on their surface
- have the chromatin (DNA and protein) in their nucleus degraded
- have their mitochondria break down with the release of cytochrome c
- break into small, membrane-wrapped, fragments
- release (at least in mammalian cells) ATP and UTP
- These nucleotides bind to receptors on wandering phagocytic cells like macrophages and dendritic cells and attract them to the dying cells (a "**find-me**" signal")
- The phospholipid **phosphatidylserine**, which is normally hidden in the inner layer of the plasma membrane, is exposed on the surface
- This "**eat me**" signal is bound by other receptors on the phagocytes which then engulf the cell fragments
- The phagocytic cells secrete cytokines that **inhibit** inflammation (e.g., IL-10 and TGF-β)

The pattern of events in death by suicide is so orderly that the process is often called **programmed cell death** or **PCD**. The cellular machinery of programmed cell death turns out to be as intrinsic to the cell as, say, mitosis. Programmed cell death is also called *apoptosis*. (There is no consensus yet on how to pronounce it; some say *APE oh TOE sis*; some say *uh POP tuh sis*.)

Why should a cell commit suicide?

There are two different reasons.

1. Programmed cell death is as needed for proper development as mitosis is.

Examples:

- The resorption of the tadpole tail at the time of its metamorphosis into a frog occurs by apoptosis.
- The formation of the fingers and toes of the fetus requires the removal, by apoptosis, of the tissue between them.
- The sloughing off of the inner lining of the uterus (the endometrium) at the start of menstruation occurs by apoptosis.
- The formation of the proper connections (synapses) between neurons in the brain requires that surplus cells be eliminated by apoptosis.
- The elimination of T cells that might otherwise mount an autoimmune attack on the body occurs by apoptosis.
- During the pupal stage of insects that undergo complete metamorphosis, most of the cells of the larva die by apoptosis thus providing the nutrients for the development of the structures of the adult.

2. Programmed cell death is needed to destroy cells that represent a threat to the integrity of the organism.

Examples:

Cells infected with viruses

One of the methods by which **cytotoxic T lymphocytes** (CTLs) kill virus-infected cells is by inducing apoptosis

and some viruses mount countermeasures to thwart it.

Cells of the immune system

As cell-mediated immune responses wane, the effector cells must be removed to prevent them from attacking body constituents. CTLs induce apoptosis in each other and even in themselves. Defects in the apoptotic machinery is associated with autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis.

Cells with DNA damage

Damage to its genome can cause a cell

- to disrupt proper embryonic development leading to birth defects
- to become cancerous.

Cells respond to DNA damage by increasing their production of p53. p53 is a potent **inducer** of apoptosis. Is it any wonder that mutations in the *p53* gene, producing a defective protein, are so often found in cancer cells (that represent a lethal threat to the organism if permitted to live)?

Cancer cells

Radiation and chemicals used in cancer therapy induce apoptosis in some types of cancer cells.

What makes a cell decide to commit suicide?

The balance between the withdrawal of positive signals; that is, signals needed for continued survival, and the receipt of negative signals.

Withdrawal of positive signals

The continued survival of most cells requires that they receive continuous stimulation from other cells and, for many, continued adhesion to the surface on which they are growing. Some examples of positive signals: growth factors for neurons and Interleukin-2 (**IL-2**), an essential factor for the mitosis of lymphocytes

Receipt of negative signals

- increased levels of oxidants within the cell
- damage to DNA by these oxidants or other agents like ultraviolet light, X-rays and chemotherapeutic drugs
- accumulation of proteins that failed to fold properly into their proper tertiary structure
- molecules that bind to specific receptors on the cell surface and signal the cell to begin the apoptosis program. These **death activators** include:
  - **Tumor necrosis factor-alpha** (**TNF-α**) that binds to the **TNF receptor**
  - **Lymphotoxin** (also known as TNF-β) that also binds to the **TNF receptor**
  - **Fas ligand** (**FasL**), a molecule that binds to a cell-surface receptor named **Fas** (also called **CD95**)

The Mechanisms of Apoptosis

There are 3 different mechanisms by which a cell commits suicide by apoptosis.

1. Generated by signals arising within the cell
2. Triggered by **death activators** binding to receptors at the cell surface:
   - TNF-α
   - Lymphotoxin
   - Fas ligand (**FasL**)
3. Triggered by dangerous reactive oxygen species

Apoptosis triggered by internal signals

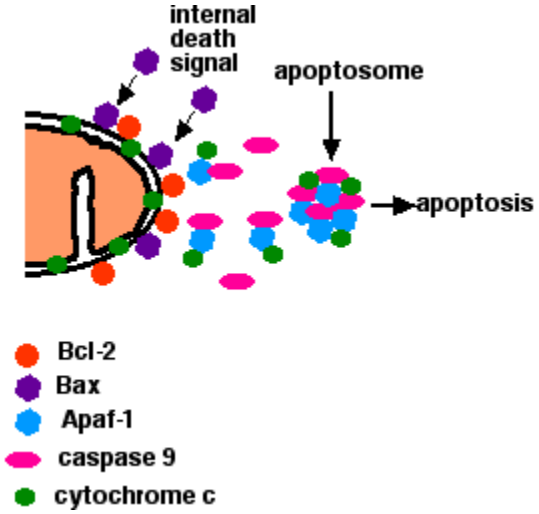


Figure 3.20.1 Apoptosis by internal trigger

- In a healthy cell, the outer membranes of its mitochondria display the protein **Bcl-2** on their surface. Bcl-2 **inhibits** apoptosis.
- Internal damage to the cell
  - causes a related protein, **Bax**, to migrate to the surface of the mitochondrion where it inhibits the protective effect of Bcl-2 and inserts itself into the outer mitochondrial membrane punching holes in it and causing
  - **cytochrome c** to leak out.
- The released cytochrome c binds to the protein **Apaf-1** ("apoptotic protease activating factor-1").
- Using the energy provided by ATP, these complexes aggregate to form **apoptosomes**. The apoptosomes bind to and activate **caspase-9**. Caspase-9 is one of a family of over a dozen caspases. They are all proteases. They get their name because they cleave proteins — mostly each other — at aspartic acid (Asp) residues.
- Caspase-9 cleaves and, in so doing, activates other caspases (caspase-3 and -7).
- The activation of these "executioner" caspases creates an expanding cascade of proteolytic activity (rather like that in blood clotting and complement activation) which leads to
  - digestion of structural proteins in the cytoplasm,
  - degradation of chromosomal DNA
  - phagocytosis of the cell

Apoptosis triggered by external signals

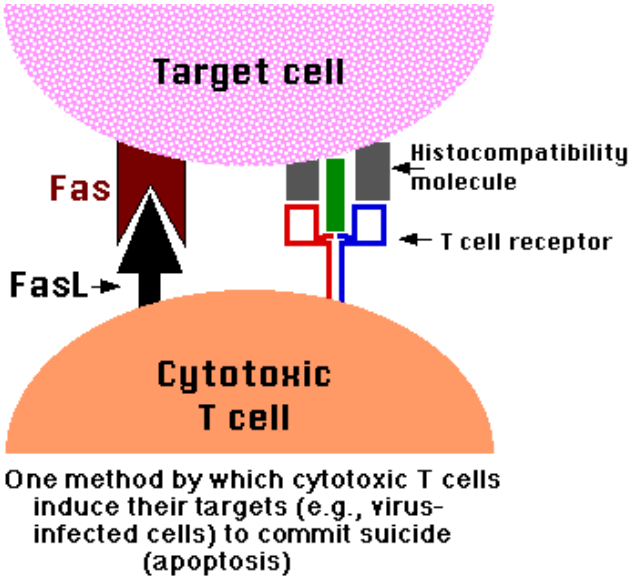


Figure 3.20.2 Apoptosis by external trigger

- **Fas** and the **TNF receptor** are integral membrane proteins with their receptor domains exposed at the surface of the cell
- Binding of the complementary **death activator** (**FasL** and **TNF** respectively) transmits a signal to the cytoplasm that leads to the activation of **caspase 8**
- Caspase 8 (like caspase 9) initiates a cascade of caspase activation leading to **phagocytosis** of the cell.
- **Example**: When cytotoxic T cells recognize (bind to) their target,
  - They produce more **FasL** at their surface.
  - This binds with the **Fas** on the surface of the target cell leading to its death by apoptosis.

The early steps in apoptosis are reversible — at least in C. elegans. In some cases, final destruction of the cell is guaranteed only with its engulfment by a phagocyte.

Apoptosis-Inducing Factor (AIF)

Neurons, and perhaps other cells, have another way to self-destruct that — unlike the two paths described above — does not use caspases. Apoptosis-inducing factor (**AIF**) is a protein that is normally located in the intermembrane space of mitochondria. When the cell receives a signal telling it that it is time to die, AIF is released from the mitochondria (like the release of cytochrome c in the first pathway). It migrates into the nucleus and binds to DNA, which triggers the destruction of the DNA and cell death.

Apoptosis and Cancer

Some viruses associated with cancers use tricks to prevent apoptosis of the cells they have transformed.

- Several **human papilloma viruses** (**HPV**) have been implicated in causing cervical cancer. One of them produces a protein (E6) that binds and inactivates the apoptosis promoter **p53**.
- **Epstein-Barr Virus** (**EBV**), the cause of mononucleosis and associated with some lymphomas
  - produces a protein similar to Bcl-2
  - produces another protein that causes the cell to increase its own production of Bcl-2. Both these actions make the cell more resistant to apoptosis (thus enabling a cancer cell to continue to proliferate).

Even cancer cells produced without the participation of viruses may have tricks to avoid apoptosis.

- Some **B-cell leukemias** and lymphomas express high levels of **Bcl-2**, thus blocking apoptotic signals they may receive. The high levels result from a translocation of the *BCL-2* gene into an **enhancer** region for antibody production.
- **Melanoma** (the most dangerous type of skin cancer) cells avoid apoptosis by inhibiting the expression of the gene encoding **Apaf-1**.
- Some cancer cells, especially lung and colon cancer cells, secrete elevated levels of a soluble "decoy" molecule that binds to FasL, plugging it up so it cannot bind Fas. Thus, cytotoxic T cells (CTL) cannot kill the cancer cells by the mechanism shown above.
- Other cancer cells express high levels of **FasL**, and can kill any cytotoxic T cells (CTL) that try to kill them because CTL also express Fas (but are protected from their own FasL).

Apoptosis in the Immune System

The immune response to a foreign invader involves the proliferation of lymphocytes — T and/or B cells. When their job is done, they must be removed leaving only a small population of memory cells. This is done by apoptosis. Very rarely humans are encountered with genetic defects in apoptosis. The most common one is a mutation in the gene for Fas, but mutations in the gene for FasL or even one of the caspases are occasionally seen. In all cases, the genetic problem produces **autoimmune lymphoproliferative syndrome** or ALPS.

Features

- an accumulation of lymphocytes in the lymph nodes and spleen greatly enlarging them.
- the appearance of clones that are autoreactive; that is, attack "self" components producing such autoimmune disorders as
  - hemolytic anemia
  - thrombocytopenia
- the appearance of **lymphoma** — a cancerous clone of lymphocytes.

In most patients with ALPS, the mutation is present in the germline; that is, every cell in their body carries it. In a few cases, however, the mutation is somatic; that is, has occurred in a precursor cell in the bone marrow. These later patients are genetic mosaics — with some lymphocytes that undergo apoptosis normally and others that do not. The latter tend to out-compete the former and grow to become the major population in the lymph nodes and blood.

Apoptosis and Organ Transplants

For many years it has been known that certain parts of the body such as the anterior chamber of the eye and the testes are "immunologically privileged sites". Antigens within these sites fail to elicit an immune response. It turns out that cells in these sites differ from the other cells of the body in that they express high levels of **FasL** at all times. Thus antigen-reactive T cells, which express **Fas**, would be killed when they enter these sites. (This is the reverse of the mechanism described above.)

This finding raises the possibility of a new way of preventing graft rejection. If at least some of the cells on a transplanted kidney, liver, heart, etc. could be made to express high levels of FasL, that might protect the graft from attack by the T cells of the host's cell-mediated immune system. If so, then the present need for treatment with immunosuppressive drugs for the rest of the transplant recipient's life would be reduced or eliminated. So far, the results in animal experiments have been mixed. Allografts engineered to express FasL have shown increased survival for kidneys, but not for hearts or islets of Langerhans.

Apoptosis in Plants

Plants, too, can turn on a system of programmed cell death; for example, in an attempt to halt the spread of virus infection. The mechanism differs from that in animals although it, too, involves a protease that — like caspases — cleaves other proteins at Asp (and Asn) residues. Activation of this enzyme destroys the central vacuole, which is followed by disintegration of the rest of the cell.

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3.21: Collagens

Collagens are insoluble, extracellular glycoproteins found in all animals. They are the most abundant proteins in the human body and are essential structural components of all connective tissues such as cartilage, bone, tendons, ligaments, fascia, skin. Gelatin is solubilized collagen. **29 types** of collagens have been found in humans and the major ones are:

- **Type I**. The chief component of tendons, ligaments, and bones.
- **Type II**. Represents more than 50% of the protein in **cartilage** and is the major component of the vitreous body of the eye. It is also used to build the notochord of vertebrate embryos.
- **Type III**. Strengthens the walls of hollow structures like arteries, the intestine, and the uterus.
- **Type IV**. Forms the **basal lamina** of epithelia. (The basal lamina is often called the **basement membrane**, but is not related to lipid bilayer membranes.) A meshwork of Type IV collagens provides the filter for the blood **capillaries** and the glomeruli of the kidneys.

The other 25 types are probably equally important, but they are much less abundant.

Collagens Structure

The basic unit of collagens is a polypeptide consisting of the repeating sequence

n

where **X** is often proline (Pro) and **Y** is often **hydroxyproline** (proline to which an -OH group is added after synthesis of the polypeptide). The resulting molecule twists into an elongated, left-handed helix (NOT an alpha helix).

A single collagen molecule, tropocollagen, is used to make up larger collagen aggregates, such as fibrils. It is approximately 300 nm long and 1.5 nm in diameter, and it is made up of three polypeptide strands (called alpha peptides, see step 2), each of which has the conformation of a left-handed helix (Figure 3.21.1). These three left-handed helices are twisted together into a right-handed triple helix or "super helix", a cooperative quaternary structure stabilized by many hydrogen bonds.

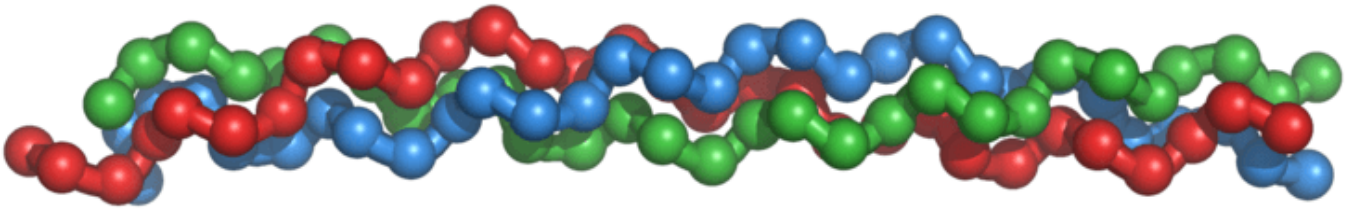


Figure 3.21.1: Tropocollagen molecule: three left-handed procollagens (red, green, blue) join to form a right handed triple helical tropocollagen. Image usd with permision (CC-SA-BY-3.0: Vossman and JWSchmidt)

When synthesized, the N- terminal and C- terminal of the polypeptide have globular domains, which keep the molecule soluble. As they pass through the endoplasmic reticulum (ER) and Golgi apparatus,

- The molecules are glycosylated.
- Hydroxyl (-OH) groups are added to the "Y" amino acid.
- S-S bonds link three chains covalently.
- The three molecules twist together to form a **triple helix**.

In some collagens (e.g., Type II), the three molecules are identical (the product of a single gene). In other collagens (e.g., Type I), two polypeptides of one kind (gene product) assemble with a second, quite similar, polypeptide, that is the product of a second gene.

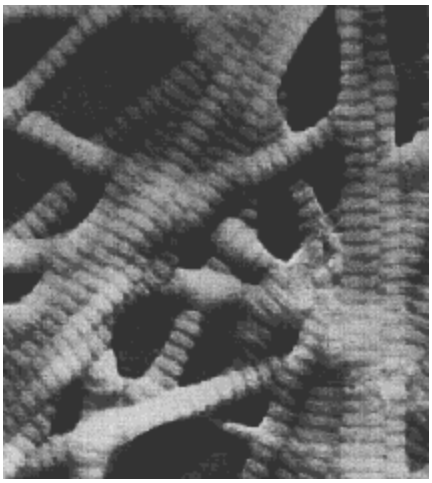


Figure 3.21.2: Collagen courtesy of Dr. Jerome Gross

When the triple helix is secreted from the cell (usually by a fibroblast), the globular ends are cleaved off. The resulting linear, insoluble molecules assemble into collagen **fibers**. They assemble in a staggered pattern that gives rise to the striations seen in the above electron micrograph. Type IV collagens are an exception; they form a meshwork rather than striated fibers.

Inherited Diseases Caused by Mutant Collagen Genes

- **Brittle-bone disease ("osteogenesis imperfecta"):** Caused by a mutation in one or the other of the two genes whose products are used to make **Type I** collagen. Like all the inherited collagen diseases, this one is inherited as a **dominant trait**. The reason: even though one collagen allele is normal, the assembly of the normal gene product with the mutant product produces defective collagen fibers. Bone marrow stem cells from patients with this disease have had their mutant gene knocked out by gene targeting and gained the ability to make good collagen and bone (when the cells were placed in immunodeficient mice). So this disease now seems to be a promising candidate for gene therapy.
- **Forms of dwarfism:** Caused by mutations in a **Type II** collagen gene.
- **Rubber-man syndrome:** Caused by a mutations in a **Type I** collagen gene. The subject has hyperextensible joints, tendons, and skin. (This inherited disorder represents one type of Ehlers-Danlos syndrome.)
- **Ehlers-Danlos syndrome:** It is caused by mutations in the gene for **Type III** collagen. Patients are at risk of rupture of major arteries or the intestine.
- **Alport's syndrome:** Most cases involve mutations in the gene on the X chromosome for one of the chains of **Type IV** collagen. So it shows the typical pattern of X-linked inheritance. Other cases are caused by two mutant autosomal genes for another of the Type IV collagen chains. Patients usually have damage to their glomeruli, leading to blood in their urine and, often, become deaf as well.
- **Herniated discs between the vertebrae?:** A study in Finland has found that some families that share a tendency to develop herniated discs (leading to sciatica) have an inherited point mutation in the gene (*COL9A2*) encoding one of the alpha chains in collagen IX. This collagen is one component of the extracellular matrix in the padding (discs) between our vertebrae.

Other Collagen Diseases

- **Scurvy**: Caused by a deficiency of vitamin C. The sufferer is unable to add hydroxyl (-OH) groups to proline to convert it into hydroxyproline.
- **Goodpasture's Syndrome:** Some people develop antibodies against an epitope on their **Type IV** collagen molecules. These attach to the basal lamina of epithelial cells and "fix" complement which damages the basal lamina. So Goodpasture's syndrome is an example of an autoimmune disorder.

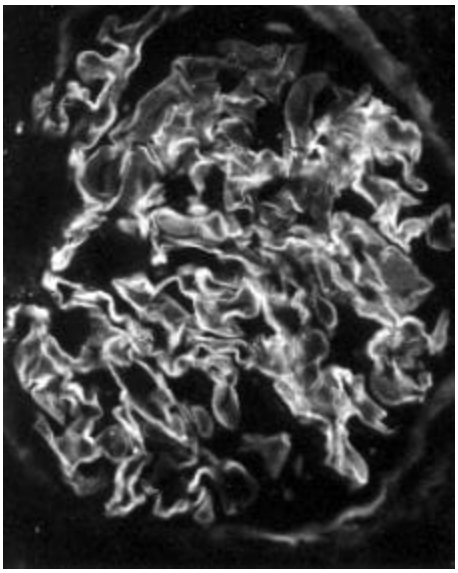


Figure 3.21.2: Goodpasture's syndrome courtesy of Dr. Frank J. Dixon

The basal lamina of the lung epithelia and the glomeruli of the kidney are especially likely to be affected. In this photo (courtesy of Dr. Frank J. Dixon), a fluorescent antibody against human IgG shows the autoantibodies coating the basement membranes of the glomeruli in a patient with Goodpasture's syndrome.

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3.22: Chromatophores

Chromatophores are irregularly shaped, pigment-containing cells. If the pigment is melanin, they are called **melanophores**. Chromatophores are common in crustaceans, cephalopod mollusks, lizards and amphibians, and some fishes.

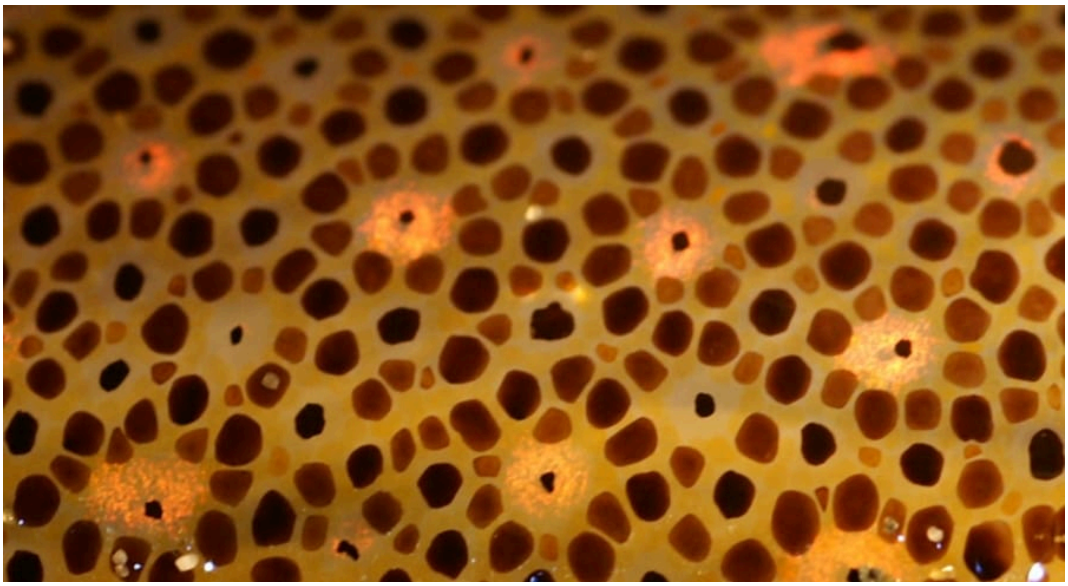


Figure 3.22.1: Chromatophores in the skin of a squid. (CC-By-Sa-2.0 Minette)

Chromatophores are often used for camouflage. Figure 3.22.1 shows a winter flounder resting on a checkerboard pattern. The chromatophores of cephalopods change size (expand and contract) as a result of activity of muscle fibers and the motor neurons that terminate at them. In crustaceans and amphibians, the chromatophores have a fixed shape. Color change comes about through the dispersal (darkening) or aggregation (lightening) of granules within the cell. This is under hormonal control.

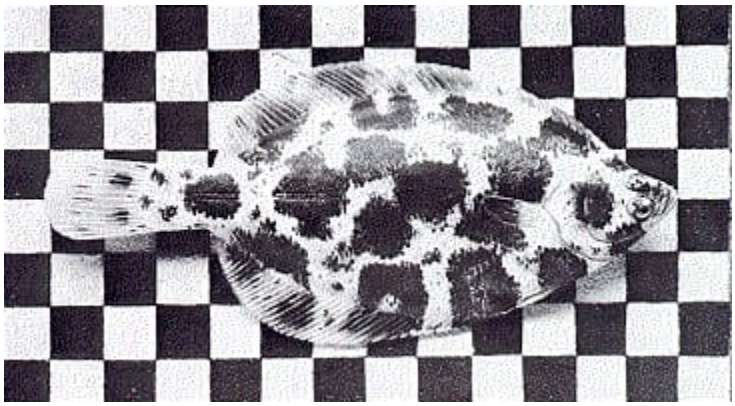


Figure 3.22.2: Chromatopores of a Flounder courtesy of the Field Museum of Natural History

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3.23: Diffusion, Active Transport and Membrane Channels

Transport Across Cell Membranes

All cells acquire the molecules and ions they need from their surrounding **extracellular fluid** (**ECF**). There is an unceasing traffic of molecules and ions in and out of the cell through its plasma membrane (Examples: glucose, Na^+, Ca^{2+}). In eukaryotic cells, there is also transport in and out of membrane-bounded intracellular compartments such as the nucleus, endoplasmic reticulum, and mitochondria (Examples: proteins, mRNA, Ca^{2+}, and ATP).

The following problems can occur during transport:

1. Relative concentrations

Molecules and ions move spontaneously down their concentration gradient (i.e., from a region of higher to a region of lower concentration) by **diffusion**. Molecules and ions can be moved **against** their concentration gradient, but this process, called **active transport**, requires the expenditure of energy (usually from ATP).

2. Lipid bilayers are impermeable to most essential molecules and ions.

The lipid bilayer is permeable to **water** molecules and a few other small, uncharged, molecules like oxygen (O_2) and carbon dioxide (CO_2). These diffuse freely in and out of the cell. The diffusion of water through the plasma membrane is of such importance to the cell that it is given a special name - **osmosis**. Lipid bilayers are **not** permeable to **ions** such as K^+, Na^+, Ca^{2+} (called **cations** because when subjected to an electric field they migrate toward the cathode [the negatively-charged electrode]) and Cl^-, HCO_3^- (called **anions** because they migrate toward the anode [the positively-charged electrode]). They are also not permeable to small hydrophilic **molecules** like glucose and **macromolecules** like proteins and RNA. The cells solve the problem of transporting ions and small molecules across their membranes with the help of the following two mechanisms:

- **Facilitated diffusion:** Transmembrane proteins create a water-filled pore through which ions and some small hydrophilic molecules can pass by diffusion. The channels can be opened (or closed) according to the needs of the cell.
- **Active transport**: Transmembrane proteins, called transporters, use the energy of ATP to force ions or small molecules through the membrane **against** their concentration gradient.

Facilitated Diffusion of Ions

Facilitated diffusion of ions takes place through proteins, or assemblies of proteins, embedded in the plasma membrane. These transmembrane proteins form a water-filled channel through which the ion can pass **down** its concentration gradient. The transmembrane channels that permit facilitated diffusion can be opened or closed. They are said to be **"gated"**; some types of gated ion channels:

- ligand-gated
- mechanically-gated
- voltage-gated
- light-gated

Ligand-gated ion channels

Many ion channels open or close in response to binding a small signaling molecule or **"ligand"**. Some ion channels are gated by extracellular ligands; some by intracellular ligands. In both cases, the ligand is **not** the substance that is transported when the channel opens.

External ligands

External ligands (shown here in green) bind to a site on the extracellular side of the channel.

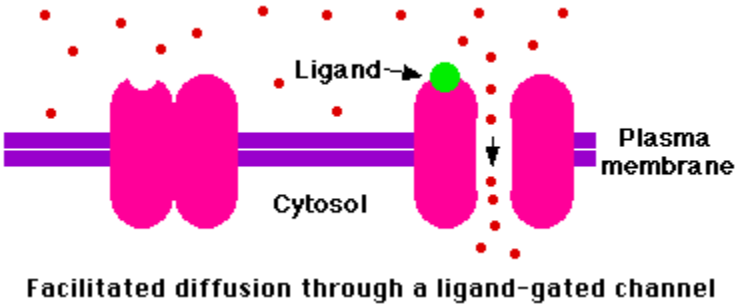


Figure 3.23.1 External Ligands

Examples:

- **Acetylcholine** (**ACh**). The binding of the neurotransmitter acetylcholine at certain synapses opens channels that admit Na^+ and initiate a nerve impulse or muscle contraction.
- **Gamma amino butyric acid** (**GABA**). Binding of GABA at certain synapses — designated $GABA_A$ — in the central nervous system admits Cl^- ions into the cell and inhibits the creation of a nerve impulse

Internal ligands

Internal ligands bind to a site on the channel protein exposed to the cytosol. **Examples:**

- "Second messengers", like **cyclic AMP** (**cAMP**) and **cyclic GMP** (**cGMP**), regulate channels involved in the initiation of impulses in neurons responding to odors and light respectively.
- **ATP** is needed to open the channel that allows chloride (Cl^-) and bicarbonate ($HCO3^-$) ions out of the cell. This channel is defective in patients with **cystic fibrosis**. Although the energy liberated by the hydrolysis of ATP is needed to open the channel, this is **not** an example of active transport; the ions diffuse through the open channel following their concentration gradient.

Mechanically-gated ion channels

Sound waves bending the cilia-like projections on the hair cells of the inner ear open up ion channels leading to the creation of nerve impulses that the brain interprets as sound. Mechanical deformation of the cells of stretch receptors opens ion channels leading to the creation of nerve impulses.

Voltage-gated ion channels

In so-called "excitable" cells like **neurons** and **muscle cells**, some channels open or close in response to changes in the charge (measured in volts) across the plasma membrane. **For example,** as an impulse passes down a neuron, the reduction in the voltage opens sodium channels in the adjacent portion of the membrane. This allows the influx of Na^+ into the neuron and thus the continuation of the nerve impulse. Some 7000 sodium ions pass through each channel during the brief period (about 1 millisecond) that it remains open. This was learned by use of the patch clamp technique.

The Patch Clamp Technique

The properties of ion channels can be studied by means of the patch clamp technique. A very fine pipette (with an opening of about 0.5 µm) is pressed against the plasma membrane of either an intact cell or the plasma membrane can be pulled away from the cell and the preparation placed in a test solution of desired composition. Current flow through a single ion channel can then be measured.

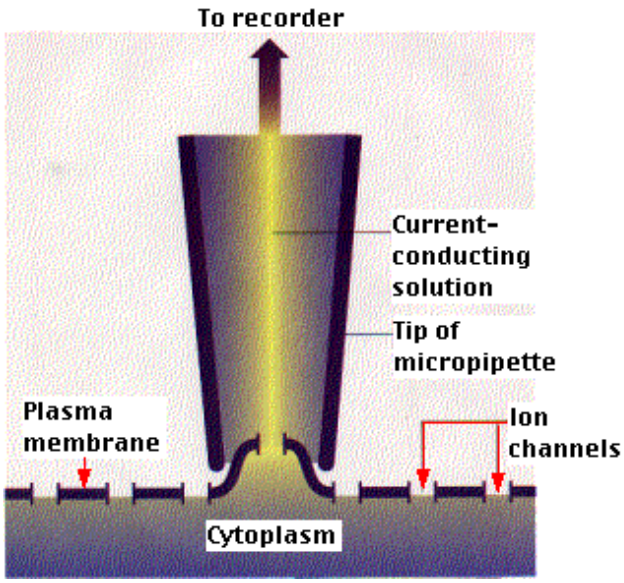


Figure 3.23.2 The Patch Clamp

Such measurements reveal that each channel is either fully open or fully closed; that is, facilitated diffusion through a single channel is "all-or-none". This technique has provided so much valuable information about ion channels that its inventors, Erwin Neher and Bert Sakmann, were awarded a Nobel Prize in 1991.

Facilitated Diffusion of Molecules

Some small, hydrophilic organic molecules, like sugars, can pass through cell membranes by facilitated diffusion. Once again, the process requires transmembrane proteins. In some cases, these — like ion channels — form water-filled pores that enable the molecule to pass in (or out) of the membrane following its concentration gradient.

Example:

Maltoporin. This homotrimer in the outer membrane of *E. coli* forms pores that allow the disaccharide maltose and a few related molecules to diffuse into the cell.

Example: The plasma membrane of human red blood cells contain transmembrane proteins that permit the diffusion of glucose from the blood into the cell.

Note that in all cases of facilitated diffusion through channels, the channels are **selective**; that is, the structure of the protein admits only certain types of molecules through. Whether all cases of facilitated diffusion of small molecules use channels is yet to be proven. Perhaps some molecules are passed through the membrane by a conformational change in the shape of the transmembrane protein when it binds the molecule to be transported.

In either case, the interaction between the molecule being transported and its transporter resembles in many ways the interaction between an enzyme and its substrate.

Active Transport

Active transport is the pumping of molecules or ions through a membrane **against** their concentration gradient. It requires a transmembrane protein (usually a complex of them) called a transporter and energy. The source of this energy is **ATP**.

The energy of ATP may be used directly or indirectly.

- **Direct Active Transport.** Some transporters bind ATP directly and use the energy of its hydrolysis to drive active transport.
- **Indirect Active Transport.** Other transporters use the energy already stored in the gradient of a directly-pumped **ion**. Direct active transport of the ion establishes a concentration gradient. When this is relieved by facilitated diffusion, the energy

released can be harnessed to the pumping of some other ion or molecule.

Direct Active Transport

The Na^+/K^+ ATPase

The cytosol of animal cells contains a concentration of potassium ions (K^+) as much as 20 times higher than that in the extracellular fluid. Conversely, the extracellular fluid contains a concentration of sodium ions (Na^+) as much as 10 times greater than that within the cell. These concentration gradients are established by the active transport of both ions. And, in fact, the same transporter, called the Na^+/K^+ ATPase, does both jobs. It uses the energy from the hydrolysis of ATP to

- actively transport 3 Na^+ ions out of the cell
- for each 2 K^+ ions pumped into the cell.

This accomplishes several vital functions:

- It helps establish a net charge across the plasma membrane with the interior of the cell being negatively charged with respect to the exterior. This **resting potential** prepares nerve and muscle cells for the propagation of action potentials leading to nerve impulses and muscle contraction.
- The accumulation of sodium ions outside of the cell draws water out of the cell and thus enables it to maintain osmotic balance (otherwise it would swell and burst from the inward diffusion of water).
- The gradient of sodium ions is harnessed to provide the energy to run several types of indirect pumps.

The crucial roles of the Na^+/K^+ ATPase are reflected in the fact that almost one-third of all the energy generated by the mitochondria in animal cells is used just to run this pump.

The H^+/K^+ ATPase

The parietal cells of your stomach use this pump to secrete gastric juice. These cells transport protons (H^+) from a concentration of about 4×10^{-8} M within the cell to a concentration of about 0.15 M in the gastric juice (giving it a pH close to 1). Small wonder that parietal cells are stuffed with mitochondria and uses huge amounts of **ATP** as they carry out this three-million fold concentration of protons.

The Ca^{2+} ATPases

A Ca^{2+} ATPase is located in the **plasma membrane** of all eukaryotic cells. It uses the energy provided by one molecule of ATP to pump one Ca^{2+} ion out of the cell. The activity of these pumps helps to maintain the ~20,000-fold concentration gradient of Ca^{2+} between the cytosol (~ 100 nM) and the ECF (~ 20 mM). In resting skeletal muscle, there is a much higher concentration of calcium ions (Ca^{2+}) in the sarcoplasmic reticulum than in the cytosol. Activation of the muscle fiber allows some of this Ca^{2+} to pass by facilitated diffusion into the cytosol where it triggers contraction.

After contraction, this Ca^{2+} is pumped back into the sarcoplasmic reticulum. This is done by another Ca^{2+} ATPase that uses the energy from each molecule of ATP to pump 2 Ca^{2+} ions.

Pumps **1. - 3.** are designated **P-type ion transporters** because they use the same basic mechanism: a conformational change in the proteins as they are reversibly phosphorylated by ATP. And all three pumps can be made to run backward. That is, if the pumped ions are allowed to diffuse back through the membrane complex, ATP can be synthesized from ADP and inorganic phosphate.

ABC Transporters

ABC ("**A**TP-**B**inding **C**assette") transporters are transmembrane proteins that

- expose a ligand-binding domain at one surface and a
- ATP-binding domain at the other surface.

The ligand-binding domain is usually restricted to a single type of molecule.

The ATP bound to its domain provides the energy to pump the ligand across the membrane.

The human genome contains 48 genes for ABC transporters. Some examples:

- **CFTR** — the cystic fibrosis transmembrane conductance regulator
- **TAP**, the transporter associated with antigen processing
- The transporter that liver cells use to pump the salts of bile acids out into the bile.
- ABC transporters that pump chemotherapeutic drugs out of cancer cells thus reducing their effectiveness.

ABC transporters must have evolved early in the history of life. The ATP-binding domains in archaea, eubacteria, and eukaryotes all share a homologous structure, the ATP-binding "cassette".

Indirect Active Transport

Indirect active transport uses the downhill flow of an ion to pump some other molecule or ion against its gradient. The driving ion is usually sodium (Na^+) with its gradient established by the Na^+/K^+ ATPase.

Symport Pumps

In this type of indirect active transport, the driving ion (Na^+) and the pumped molecule pass through the membrane pump in the **same** direction. Examples:

- **The Na^+/glucose transporter**. This transmembrane protein allows sodium ions and glucose to enter the cell together. The sodium ions flow **down** their concentration gradient while the glucose molecules are pumped **up** theirs. Later the sodium is pumped back out of the cell by the Na^+/K^+ ATPase. The Na^+/glucose transporter is used to actively transport glucose out of the intestine and also out of the kidney tubules and back into the blood.
- All the **amino acids** can be actively transported, for example out of the kidney tubules and into the blood, by sodium-driven symport pumps.
- Sodium-driven symport pumps also return **neurotransmitters** to the presynaptic neuron.
- **The Na^+/iodide transporter**. This symporter pumps iodide ions into the cells of the thyroid gland (for the manufacture of thyroxine) and also into the cells of the mammary gland (to supply the baby's need for iodide).
- The **permease** encoded by the lac operon of *E. coli* that transports lactose into the cell.

Antiport Pumps

In antiport pumps, the driving ion (again, usually sodium) diffuses through the pump in one direction providing the energy for the active transport of some other molecule or ion in the opposite direction. **Example**:

Ca^{2+} ions are pumped out of cells by sodium-driven antiport pumps. Antiport pumps in the vacuole of some plants harness the outward facilitated diffusion of protons (themselves pumped into the vacuole by a H^+ ATPase) to the active inward transport of sodium ions. This sodium/proton antiport pump enables the plant to sequester sodium ions in its vacuole. Transgenic tomato plants that overexpress this sodium/proton antiport pump are able to thrive in saline soils too salty for conventional tomatoes.

Antiport pumps to the active inward transport of nitrate ions ($NO3^-$)

Some inherited ion-channel diseases

A growing number of human diseases have been discovered to be caused by inherited mutations in genes encoding channels.

Examples:

- **Chloride-channel** diseases
  - cystic fibrosis
  - inherited tendency to kidney stones (caused by a different kind of chloride channel than the one involved in cystic fibrosis)
- **Potassium-channel** diseases
  - the majority of cases of **long QT syndrome**, an inherited disorder of the heartbeat
  - a rare, inherited tendency to epileptic seizures in the newborn
  - several types of inherited **deafness**
- **Sodium-channel** diseases
  - inherited tendency to certain types of muscle spasms
  - Liddle's syndrome. Inadequate sodium transport out of the kidneys, because of a mutant sodium channel, leads to elevated **osmotic pressure** of the blood and resulting hypertension (high blood pressure)

Osmosis

Osmosis is a special term used for the diffusion of water through cell membranes. Although water is a polar molecule, it is able to pass through the lipid bilayer of the plasma membrane. Aquaporins — transmembrane proteins that form hydrophilic channels — greatly accelerate the process, but even without these, water is still able to get through. Water passes by diffusion from a region of higher to a region of lower concentration. Note that this refers to the concentration of water, NOT the concentration of any solutes present in the water. Water is never transported actively; that is, it never moves against its concentration gradient. However, the concentration of water can be altered by the active transport of solutes and in this way the movement of water in and out of the cell can be controlled. **Example**: the reabsorption of water from the kidney tubules back into the blood depends on the water following behind the active transport of Na^+.

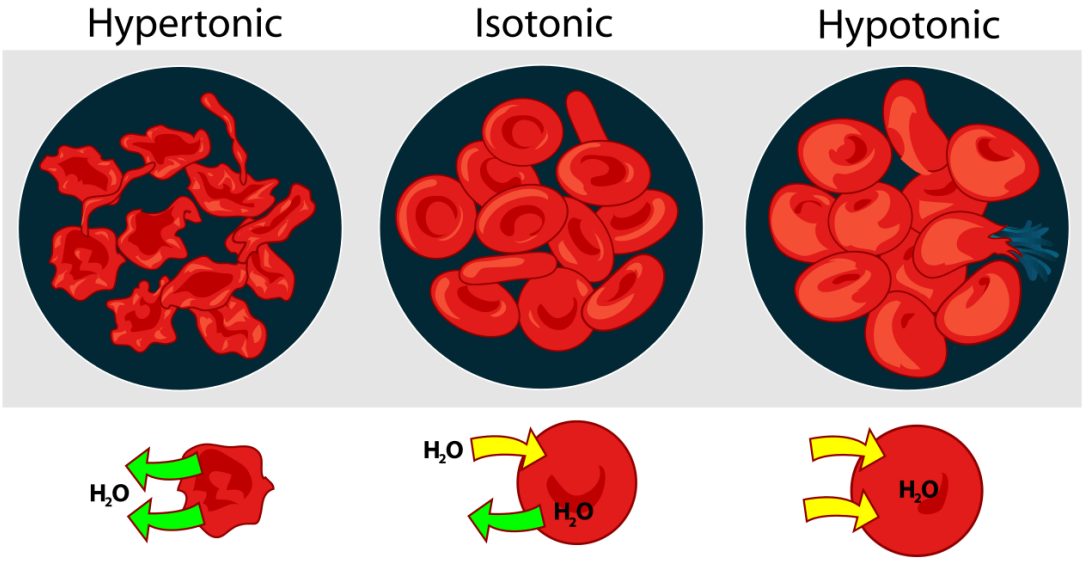


Figure 3.23.3: Osmosis effect on blood cells in different solutions. (Public domain; Ladyofhats).

- **Hypotonic solutions:** If the concentration of water in the medium surrounding a cell is greater than that of the cytosol, the medium is said to be **hypotonic**. Water enters the cell by osmosis. A red blood cell placed in a hypotonic solution (e.g., pure water) bursts immediately ("hemolysis") from the influx of water. Plant cells and bacterial cells avoid bursting in hypotonic surroundings by their strong cell walls. These allow the buildup of **turgor** within the cell. When the turgor pressure equals the osmotic pressure, osmosis ceases.
- **Isotonic solutions**: When red blood cells are placed in a 0.9% salt solution, they neither gain nor lose water by osmosis.

Such a solution is said to be **isotonic**. The extracellular fluid (ECF) of mammalian cells is isotonic to their cytoplasm. This balance must be actively maintained because of the large number of organic molecules dissolved in the cytosol but not present in the ECF. These organic molecules exert an osmotic effect that, if not compensated for, would cause the cell to take in so much water that it would swell and might even burst. This fate is avoided by pumping sodium ions out of the cell with the Na^+/K^+ ATPase.

- **Hypertonic solutions:** If red cells are placed in sea water (about 3% salt), they lose water by osmosis and the cells shrivel up. Sea water is **hypertonic** to their cytosol. Similarly, if a plant tissue is placed in sea water, the cell contents shrink away from the rigid cell wall. This is called **plasmolysis**. Sea water is also hypertonic to the ECF of most marine vertebrates. To avoid fatal dehydration, these animals (e.g., bony fishes like the cod) must continuously drink sea water and then desalt it by pumping ions out of their gills by active transport.

Marine birds, which may pass long periods of time away from fresh water, and sea turtles use a similar device. They, too, drink salt water to take care of their water needs and use metabolic energy to desalt it. In the herring gull, shown here, the salt is extracted by two glands in the head and released (in a very concentrated solution — it is saltier than the blood) to the outside through the nostrils. Marine snakes use a similar desalting mechanism.

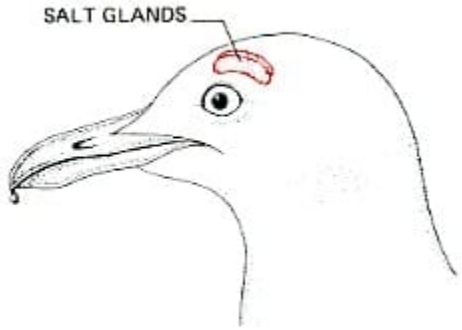


Figure 3.23.4: herring gull salt glands

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3.24: Endocytosis

In endocytosis, the cell engulfs some of its extracellular fluid (ECF) including material dissolved or suspended in it. A portion of the plasma membrane is invaginated, coated with molecules of the protein clathrin, and pinched off forming a membrane-bounded vesicle called an endosome (Figure 3.24.1).

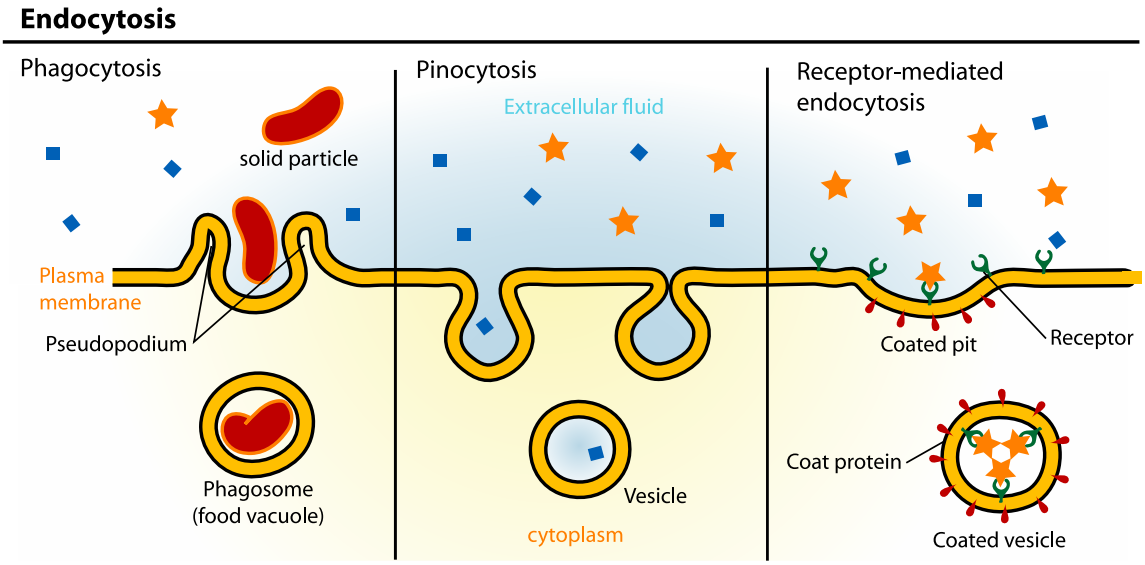


Figure 3.24.1: Endocytosis (IPA: [ɛndəʊsaɪˈtəʊsɪs]) is a process whereby cells absorb material (molecules such as proteins) from the outside by engulfing it with their cell membrane. It is used by all cells of the body because most substances important to them are polar and consist of big molecules, and thus cannot pass through the hydrophobic plasma membrane. (Public Domain; Mariana Ruiz Villarreal LadyofHats)

Phagocytosis

Phagocytosis ("cell eating") results in the ingestion of particulate matter (e.g., bacteria) from the ECF. The endosome is so large that it is called a phagosome or vacuole. Phagocytosis occurs only in certain specialized cells (e.g., neutrophils, macrophages, the amoeba) and occurs sporadically.

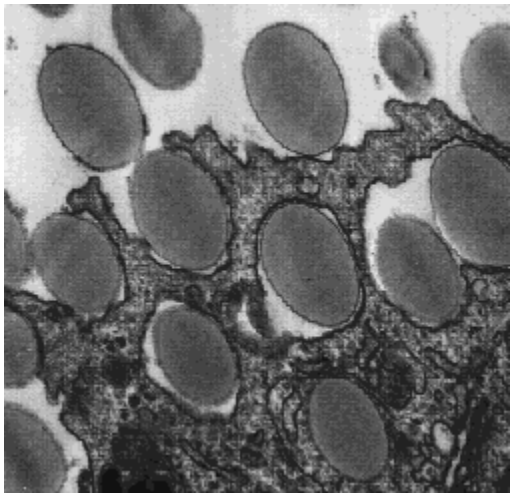


Figure 3.24.2: guinea pig Phagocyte courtesy of Dr. Robert J. North

This electron micrograph shows a guinea pig phagocyte ingesting polystyrene beads. Several beads are already enclosed in phagosomes while the others are in the process of being engulfed. In due course, phagosomes deliver their contents to

lysosomes. The membranes of the two organelles fuse. Once inside the lysosome, the contents of the phagosome, e.g. ingested bacteria, are destroyed by the degradative enzymes of the lysosome.

Games parasites play

Phagocytic cells, like macrophages and neutrophils, are an early line of defense against invading bacteria. However, some bacteria have evolved mechanisms to avoid destruction even after they have been engulfed by phagocytes.

Examples:

- ***Salmonella enterica*** is a bacterium that causes food poisoning in humans. Once engulfed by phagocytosis, it secretes a protein that prevents the fusion of its phagosome with a lysosome.
- **Mycobacteria** (e.g., the tubercle bacillus that causes tuberculosis) use a different trick.
  - When the phagosome is first pinched off from the plasma membrane, it is coated with a protein called "TACO" (for tryptophan-aspartate-containing coat protein).
  - This must be removed before the phagosome can fuse with a lysosome.
  - Mycobacteria taken into a phagosome are able, in some way, to keep the TACO coat from being removed.
  - Thus there is no fusion with lysosomes and the mycobacteria can continue to live in this protected intracellular location.

Some **intracellular** parasites exploit receptor-mediated endocytosis to sneak their way into their host cell. They have evolved surface molecules that serve as decoy ligands for receptors on the target cell surface. Binding to these receptors tricks the cell into engulfing the parasite.

Examples:

- **Epstein-Barr Virus** (**EBV**). This virus causes mononucleosis and is a contributing factor in the development of Burkitt's lymphoma, a cancer of B lymphocytes. It binds to a receptor present on the surface of B cells.
- **Influenza virus**. The **hemagglutinin** on the surface of the virus binds to carbohydrate on the surface of the target cell tricking the cell into engulfing it.
- ***Listeria monocytogenes***. This food-borne bacterium can be dangerous to people with defective immune systems as well as to pregnant women and their newborn babies. It has two kinds of surface molecules each a ligand for a different receptor on the target cell surface.
- ***Streptococcus pneumoniae***. Epithelial cells like those in the nasopharynx have receptors that are responsible for transporting IgA and IgM antibodies from the blood to the apical surface of the cell. The pneumococcus piggybacks on this receptor on its return trip into the cell. **This is the organism that led to the discovery that genes are DNA.**

Pinocytosis

In pinocytosis ("cell drinking"), the drop engulfed is relatively small (Figure $3.24.1$). Pinocytosis occurs in almost all cells and continuously.

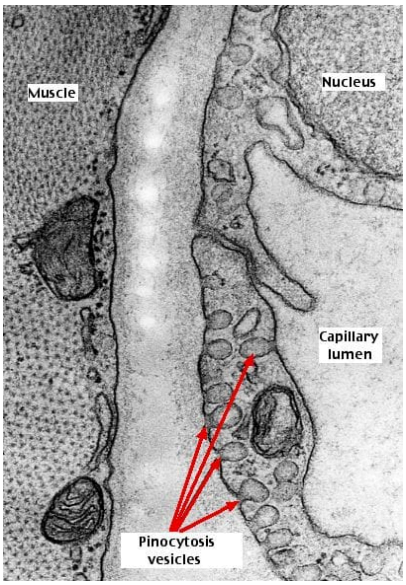


Figure 3.24.3: Pinocytosis courtesy Fawcett, The Cell: Its Organelles and Inclusions, W. B. Saunders Co., 1966

This electron micrograph shows a section of the wall of a capillary (the smallest of the blood vessels). On the right is the interior or lumen of the capillary. In the middle is the tissue space separating the capillary wall from a nearby muscle cell (left). The small inpocketings of the plasma membrane are clearly seen (arrows). Most of these are open to the tissue space but some can also be seen on the other side of the cell apparently engulfing fluid from within the capillary. Perhaps most of the vesicles facing the tissue space are not taking up material by endocytosis but are instead discharging material by exocytosis. If so, the pinocytic vesicles formed at one surface of the cell may, after being detached, move through the cell to the opposite surface and there discharge their contents. In this way materials can be moved efficiently through the capillary wall. The pinocytosis vesicles in this image represent a subtype called **caveolae**. In addition to their function in endo- and exocytosis, they also can serve as a reservoir of plasma membrane. When a cell expands (e.g., by osmotic swelling) or is stretched, the caveolae flatten out providing more plasma membrane.

A cell sipping away at the ECF by pinocytosis acquires a representative sample of the molecules and ions dissolved in the ECF. But cells also have a much more elegant method for picking up critical components of the ECF that may be in scant supply as we shall now see.

Receptor-Mediated Endocytosis

Some of the integral membrane proteins that a cell displays at its surface are receptors for particular components of the ECF (Figure 3.24.1*c*). For example, iron is transported in the blood complexed to a protein called **transferrin**. Cells have receptors for transferrin on their surface. When these receptors encounter a molecule of transferrin, they bind tightly to it. The complex of transferrin and its receptor is then engulfed by endocytosis. Ultimately, the iron is released into the cytosol. The strong **affinity** of the transferrin receptor for transferrin (its **ligand**) ensures that the cell will get all the iron it needs even if transferrin represents only a small fraction of the protein molecules present in the ECF. Receptor-mediated endocytosis is many thousand times more efficient than simple pinocytosis in enabling the cell to acquire the macromolecules it needs.

Low-Density Lipoprotein (LDL) Receptor

Cells take up cholesterol by receptor-mediated endocytosis. Cholesterol is an essential component of all cell membranes. Most cells can, as needed, either synthesize cholesterol or acquire it from the ECF. Human cells get much of their cholesterol from the liver and, if your diet is not strictly "100% cholesterol-free", by absorption from the intestine.

Cholesterol is a hydrophobic molecule and quite insoluble in water. Thus it cannot pass from the liver and/or the intestine to the cells simply dissolved in blood and ECF. Instead it is carried in tiny droplets of lipoprotein. The most abundant cholesterol carriers in humans are the **low-density lipoproteins** or **LDL**s.

LDL particles are spheres covered with a single layer of phospholipid molecules with their hydrophilic heads exposed to the

watery fluid (e.g., blood) and their hydrophobic tails directed into the interior. Some 1,500 molecules of cholesterol (each bound to a fatty acid) occupy the hydrophobic interior of LDL particles. One molecule of a protein called **apolipoprotein B** (apoB) is exposed at the surface of each LDL particle.

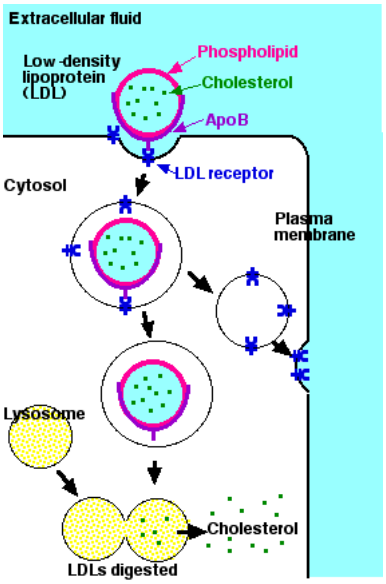


Figure 3.24.4: LDL Receptor

The first step in acquiring LDL particles is for them to bind to **LDL receptors** exposed at the cell surface. These transmembrane proteins have a site that recognizes and binds to the apolipoprotein B on the surface of the LDL. The portion of the plasma membrane with bound LDL is internalized by endocytosis. A drop in the pH (from ~7 to ~5) causes the LDL to separate from its receptor. The vesicle then pinches apart into two smaller vesicles: one containing free LDLs; the other containing now-empty receptors. The vesicle with the LDLs fuses with a lysosome to form a **secondary lysosome**. The enzymes of the lysosome then release free cholesterol into the cytosol. The vesicle with unoccupied receptors returns to and fuses with the plasma membrane, turning inside out as it does so (exocytosis). In this way the LDL receptors are returned to the cell surface for reuse.

People who inherit two defective (mutant) genes for the LDL receptor have receptors that function poorly or not at all. This creates excessively high levels of LDL in their blood and predisposes them to atherosclerosis and heart attacks. The ailment is called **familial** (because it is inherited) **hypercholesterolemia**.

Mutations in ***APOB***, the apoB gene, cause another form of inherited hypercholesterolemia.

Other small hydrophobic molecules are also transported in the blood while bound to soluble proteins:

- the retinoid vitamin A (retinol) bound to the retinol-binding protein
- the steroids
  - 25[OH] vitamin D_3 bound to the vitamin D binding protein
  - cortisol bound to the corticosteroid binding globulin
  - testosterone and estrogens bound to the sex hormone binding globulin

There is growing evidence that, like cholesterol, they are taken into the cell by receptor-mediated endocytosis.

Endocytosis removes portions of the plasma membrane and takes them inside the cell. To keep in balance, membrane must be returned to the plasma membrane. This occurs by exocytosis.

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3.25: Exocytosis

Exocytosis is the reverse of endocytosis and that is just as well. In 30 minutes an active cell like a macrophage can endocytose an amount of plasma membrane equal to its complete plasma membrane. The electron micrograph in Figure 3.25.1 shows a guinea pig phagocyte ingesting polystyrene beads. Several beads are already enclosed in vacuoles while the others are in the process of being engulfed. So the cell must have a mechanism to restore the normal amount of plasma membrane. Exocytosis is that mechanism.

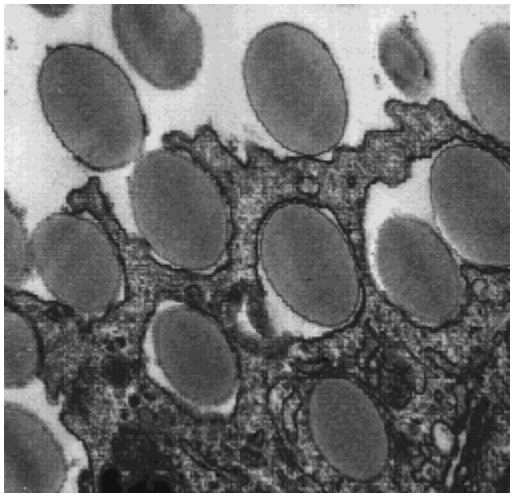


Figure 3.25.1: Guinea pig Phagocyte courtesy Dr. Robert J. North

The Secretion Mechanism

Membrane-enclosed vesicles move to the cell surface where they **fuse** with the plasma membrane. This restores the normal amount of plasma membrane and any molecules dissolved in the fluid contents of these vesicles are discharged into the extracellular fluid - this is called **secretion** (e.g., the various components of the extracellular matrix are secreted by exocytosis). Any integral membrane proteins exposed to the **interior** surface of the vesicles will now be displayed at the cell surface because the vesicles turn inside out as they fuse with the plasma membrane. Thus exocytosis does not simply replace plasma membrane, but ensures that the plasma membrane will display its **characteristic cell-surface proteins**.

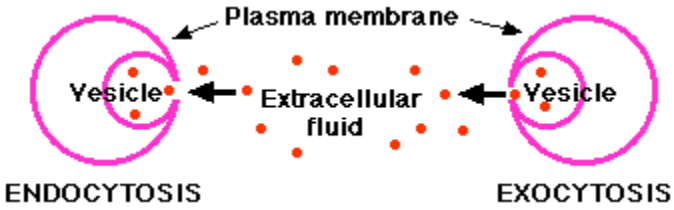


Figure 3.25.2: Exocytosis/ Endocytosis

Exocytic vesicles are created from several sources. Some are simply endosomes traversing the cell and others are pinched off from endosomes before they fuse with lysosomes. Others bud off from the endoplasmic reticulum and Golgi apparatus taking their products to the surface of the cell. The exocytosis of lysosomes supplies the membrane needed to repair wounds in the plasma membrane.

Some cells specialize in secretion. In cells that secrete large amounts of protein, for example, the protein accumulates in specialized secretory granules formed by the Golgi apparatus. These move to the cell surface and discharge their contents to the outside. For example, exocrine cells in the pancreas synthesize and secrete pancreatic digestive enzymes. The electron micrograph in Figure 3.25.4 shows four cells in the pancreas of a bat. The lumen where their apical surfaces meet leads eventually to the pancreatic duct draining into the small intestine. The spherical bodies (budded off from the Golgi apparatus) contain precursors of digestive enzymes. One is discharging its contents into the lumen by exocytosis (red arrow).

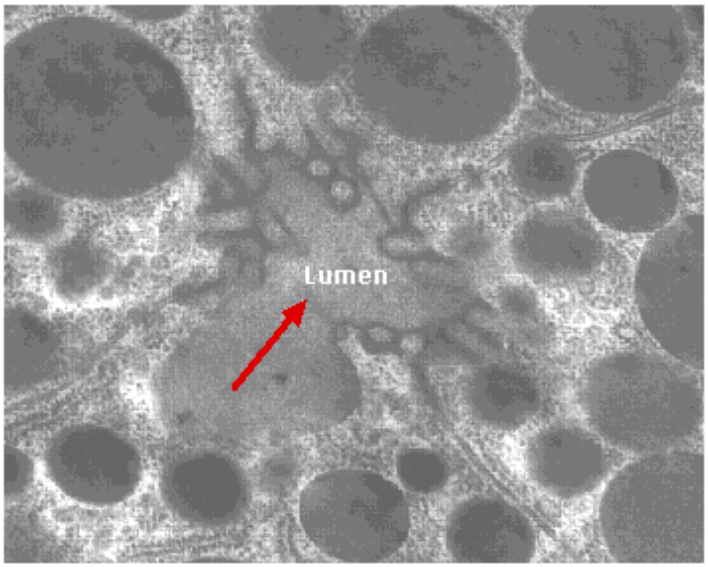


Figure 3.25.4: Pancreas cells of a bat courtesy Fawcett, The Cell: Its Organelles and Inclusions, W. B. Saunders Co., 1966

The cells lining our intestine synthesize tiny droplets of fat and discharge them into the lacteals by exocytosis.

The Kiss-and-Run Mechanism

The process described above involves the **fusion** of the exocytotic vesicle with the plasma membrane. In some cells, such as at synapses, a second type of exocytosis also takes place: (1) the vesicles make a brief contact at the plasma membrane, (2) release their contents (neurotransmitters in this case) to the exterior and (3) retreat back into the cytosol. This "kiss-and-run" version of exocytosis does not restore plasma membrane to the cell.

The Exosome Mechanism

A third type of exocytosis is found in some cells and involves endosomes themselves invaginating their membrane. As the invaginations break off they produce vesicles within vesicles, called multivesicular bodies. When these fuse with the cell's plasma membrane, these tiny (40–100 nm) internal vesicles — called **exosomes** — are secreted. Exosomes are produced in abundance by dendritic cells and B-cells and enhance their antigen-presenting function.

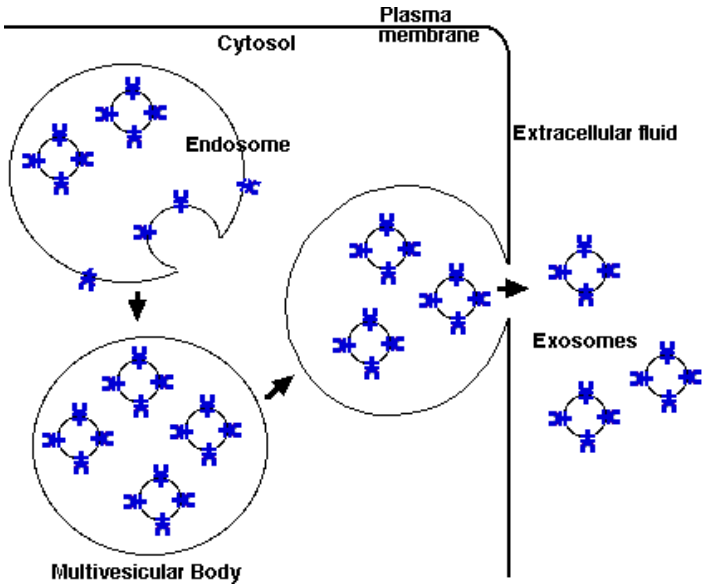


Figure 3.25.5: Exosomes

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UNIT 4: CELL METABOLISM

Metabolism is the set of life-sustaining chemical transformations within the cells of living organisms. The three main purposes of metabolism are the conversion of food/fuel to energy to run cellular processes, the conversion of food/fuel to building blocks for proteins, lipids, nucleic acids, and some carbohydrates, and the elimination of nitrogenous wastes. These enzyme-catalyzed reactions allow organisms to grow and reproduce, maintain their structures, and respond to their environments.

4.1: Enzymes

4.2: ATP

4.3: NAD and NADP

4.4: Glycolysis

4.5: Cellular Respiration

4.6: ATP Synthase

4.7: Photosynthesis - Pathway of Carbon Fixation

4.8: Photosynthesis - The Role of Light

4.9: Photosynthesis - Dicovering the Secrets

4.10: Chemiosmosis

4.11: Metabolism

4.12: Intermediary Metabolism

4.13: G Proteins

4.14: Secondary Messengers

4.15: Bioluminescence

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4.1: Enzymes

Enzymes are catalysts. Most are proteins. (A few ribonucleoprotein enzymes have been discovered and, for some of these, the catalytic activity is in the RNA part rather than the protein part. Link to discussion of these **ribozymes**). Enzymes bind temporarily to one or more of the reactants — the **substrate**(s) — of the reaction they catalyze. In doing so, they lower the amount of **activation energy** needed and thus speed up the reaction.

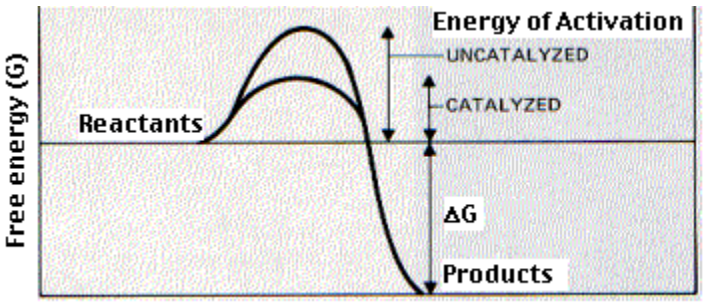


Figure 4.1.1: Activation Energy

Examples:

- **Catalase** catalyzes the decomposition of hydrogen peroxide into water and oxygen.

$$2H_2O_2 \rightarrow 2H_2O + O_2 \tag{4.1.1}$$

  One molecule of catalase can break 40 million molecules of hydrogen peroxide each second.
- **Carbonic anhydrase i**s found in red blood cells where it catalyzes the reaction.

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^- \tag{4.1.2}$$

  It enables red blood cells to transport carbon dioxide from the tissues to the lungs. One molecule of carbonic anhydrase can process one million molecules of CO_2 each second.
- **Acetylcholinesterase** catalyzes the breakdown of the neurotransmitter **acetylcholine** at several types of synapses as well as at the neuromuscular junction — the specialized synapse that triggers the contraction of skeletal muscle. One molecule of acetylcholinesterase breaks down 25,000 molecules of acetylcholine each second. This speed makes possible the rapid "resetting" of the synapse for transmission of another nerve impulse.

Enzyme activity can be analyzed quantitatively. To do its work, an enzyme must unite — even if ever so briefly — with at least one of the reactants. In most cases, the forces that hold the substrate in the **active site** of the enzyme are **noncovalent**, an assortment of hydrogen bonds, ionic interactions, and hydrophobic interactions.

Most of these interactions are weak and especially so if the atoms involved are farther than about one angstrom from each other. So successful binding of the substrate in the active site of the enzyme requires that the two molecules be able to approach each other closely over a fairly broad surface. Thus the analogy that a substrate molecule binds its enzyme like a key in a lock. This requirement for complementarity in the configuration of substrate and enzyme explains the remarkable **specificity** of most enzymes. Generally, a given enzyme is able to catalyze only a single chemical reaction or, at most, a few reactions involving substrates sharing the same general structure.

Competitive inhibition

The necessity for a close, if brief, fit between enzyme and substrate explains the phenomenon of competitive inhibition. One of the enzymes needed for the release of energy within the cell is **succinic dehydrogenase**. It catalyzes the oxidation (by the removal of two hydrogen atoms) of succinic acid. If one adds **malonic acid** to cells, or to a test tube mixture of succinic acid and the enzyme, the action of the enzyme is strongly inhibited. This is because the structure of malonic acid allows it to bind to the same site on the enzyme. But there is no oxidation so no speedy release of products. The inhibition is called competitive because if you increase the ratio of succinic to malonic acid in the mixture, you will gradually restore the rate of catalysis. At a 50:1 ratio,

the two molecules compete on roughly equal terms for the binding (=catalytic) site on the enzyme.

Enzyme cofactors

Many enzymes require the presence of an additional nonprotein - a cofactor.

- Some of these are metal ions such as Zn^{2+} (the cofactor for carbonic anhydrase), Cu^{2+}, Mn^{2+}, K^{+}, and Na^{+}.
- Some cofactors are small organic molecules called **coenzymes**. The B vitamins thiamine (B1), riboflavin (B2) and nicotinamide
  are precursors of coenzymes.

Coenzymes may be covalently bound to the protein part (called the **apoenzyme**) of enzymes as a **prosthetic group**. Others bind more loosely and, in fact, may bind only transiently to the enzyme as it performs its catalytic act.

Lysozyme: a model of enzyme action

A number of lysozymes are found in nature; in human tears and egg white, for examples. The enzyme is antibacterial because it degrades the polysaccharide that is found in the cell walls of many bacteria. It does this by catalyzing the insertion of a water molecule at forming a glycosidic bond. This hydrolysis breaks the chain at that point.

The bacterial polysaccharide consists of long chains of alternating amino sugars:

- **N-acetylglucosamine** (**NAG**)
- **N-acetylmuramic acid** (**NAM**)

These hexose units resemble glucose except for the presence of the side chains containing amino groups.

Lysozyme is a globular protein with a deep cleft across part of its surface. Six hexoses of the substrate fit into this cleft. With so many oxygen atoms in sugars, as many as 14 hydrogen bonds form between the six amino sugars and certain amino acid R groups such as **Arg-114**, **Asn-37**, **Asn-44**, **Trp-62**, **Trp-63**, and **Asp-101**. Some hydrogen bonds also form with the C=O groups of several peptide bonds. In addition, hydrophobic interactions may help hold the substrate in position.

X-ray crystallography has shown that as lysozyme and its substrate unite, each is slightly deformed. The fourth hexose in the chain (ring #4) becomes twisted out of its normal position. This imposes a strain on the C-O bond on the ring-4 side of the oxygen bridge between rings 4 and 5. It is just at this point that the polysaccharide is broken. A molecule of water is inserted between these two hexoses, which breaks the chain. Here, then, is a structural view of what it means to lower activation energy. The energy needed to break this covalent bond is lower now that the atoms connected by the bond have been distorted from their normal position.

As for lysozyme itself, binding of the substrate induces a small (~0.75Å) movement of certain amino acid residues so the cleft closes slightly over its substrate. So the "lock" as well as the "key" changes shape as the two are brought together. (This is sometimes called "induced fit".)

The amino acid residues in the vicinity of rings 4 and 5 provide a plausible mechanism for completing the catalytic act. Residue 35, glutamic acid (**Glu-35**), is about 3Å from the -O- bridge that is to be broken. The free carboxyl group of glutamic acid is a hydrogen ion donor and available to transfer H^{+} to the oxygen atom. This would break the already-strained bond between the oxygen atom and the carbon atom of ring 4.

Now having lost an electron, the carbon atom acquires a positive charge. Ionized carbon is normally very unstable, but the attraction of the negatively-charged carboxyl ion of **Asp-52** could stabilize it long enough for an -OH ion (from a spontaneously dissociated water molecule) to unite with the carbon. Even at pH 7, water spontaneously dissociates to produce H^{+} and OH^{-} ions. The hydrogen ion (H^{+}) left over can replace that lost by **Glu-35**. In either case, the chain is broken, the two fragments separate from the enzyme, and the enzyme is free to attach to a new location on the bacterial cell wall and continue its work of digesting it.

Factors Affecting Enzyme Action

The activity of enzymes is strongly affected by changes in **pH** and **temperature**. Each enzyme works best at a certain pH (left graph) and temperature (right graph), its activity decreasing at values above and below that point. This is not surprising considering the importance of tertiary structure (i.e. shape) in enzyme function and noncovalent forces, e.g., ionic interactions and hydrogen bonds, in determining that shape. **Examples**:

- the protease **pepsin** works best as a pH of 1–2 (found in the stomach)
- the protease **trypsin** is inactive at such a low pH but very active at a pH of 8 (found in the small intestine as the bicarbonate of the pancreatic fluid neutralizes the arriving stomach contents).

Changes in pH alter the state of ionization of charged amino acids (e.g., Asp, Lys) that may play a crucial role in substrate binding and/or the catalytic action itself. Without the unionized -COOH group of **Glu-35** and the ionized -COO^- of **Asp-52**, the catalytic action of lysozyme would cease. Hydrogen bonds are easily disrupted by increasing temperature. This, in turn, may disrupt the shape of the enzyme so that its affinity for its substrate diminishes.

Regulation of Enzyme Activity

Several mechanisms work to make enzyme activity within the cell efficient and well-coordinated.

Anchoring enzymes in membranes

Many enzymes are inserted into cell membranes, for examples,

- the plasma membrane
- the membranes of mitochondria and chloroplasts
- the endoplasmic reticulum
- the nuclear envelope

These are locked into spatial relationships that enable them to interact efficiently.

Inactive precursors

Enzymes, such as proteases, that can attack the cell itself are inhibited while within the cell that synthesizes them. For example, pepsin is synthesized within the chief cells (in gastric glands) as an inactive precursor, **pepsinogen**. Only when exposed to the low pH outside the cell is the inhibiting portion of the molecule removed and active pepsin produced.

Feedback Inhibition

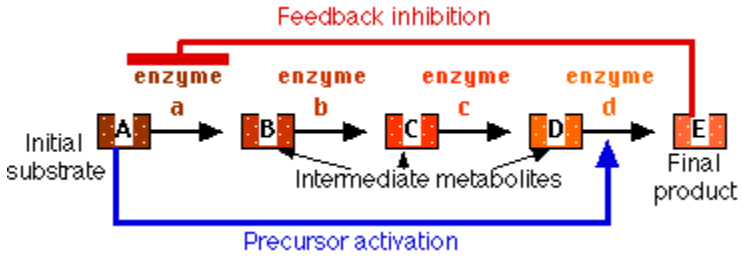


Figure 4.1.1: Feedback Inhibition

If the product of a series of enzymatic reactions, e.g., an amino acid, begins to accumulate within the cell, it may specifically inhibit the action of the first enzyme involved in its synthesis (red bar). Thus further production of the enzyme is halted.

Precursor Activation

The accumulation of a substance within a cell may specifically activate (blue arrow) an enzyme that sets in motion a sequence of

reactions for which that substance is the initial substrate. This reduces the concentration of the initial substrate.

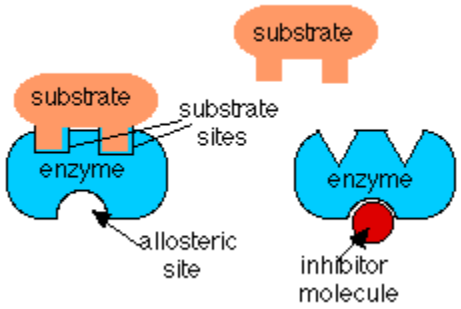


Figure 4.1.3 Precursor Activation

In the case if feedback inhibition and precursor activation, the activity of the enzyme is being regulated by a molecule which is **not** its substrate. In these cases, the regulator molecule binds to the enzyme at a different site than the one to which the substrate binds. When the regulator binds to its site, it alters the shape of the enzyme so that its activity is changed. This is called an **allosteric** effect. In feedback inhibition, the allosteric effect lowers the affinity of the enzyme for its substrate and in precursor activation, the regulator molecule increases the affinity of the enzyme in the series for its substrate.

Regulation of Enzyme Synthesis

The four mechanisms described above regulate the activity of enzymes already present within the cell. What about enzymes that are not needed or are needed but not present? Here, too, control mechanisms are at work that regulate the rate at which new enzymes are synthesized. Most of these controls work by turning on or off — the transcription of genes.

If, for example, ample quantities of an amino acid are already available to the cell from its extracellular fluid, synthesis of the enzymes that would enable the cell to produce that amino acid for itself is shut down. Conversely, if a new substrate is made available to the cell, it may induce the synthesis of the enzymes needed to cope with it. Yeast cells, for example, do not ordinarily metabolize lactose, and no **lactase** can be detected in them. However, if grown in a medium containing lactose, they soon begin synthesizing lactase — by transcribing and translating the necessary gene(s) — and so can begin to metabolize the sugar. *E. coli* also has a mechanism which regulates enzyme synthesis by controlling **translation** of a needed messenger RNA.

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4.2: ATP

ATP (Adenosine triphosphate) is a nucleotide that performs many essential roles in the cell.

- It is the major **energy currency** of the cell, providing the energy for most of the energy-consuming activities of the cell.
- It is one of the monomers used in the synthesis of **RNA** and, after conversion to deoxyATP (dATP), **DNA**.
- It regulates many biochemical pathways.

Energy

When the third phosphate group of ATP is removed by hydrolysis, a substantial amount of free energy is released. The exact amount depends on the conditions, but we shall use a value of 7.3 kcal per mole.

$$\mathrm{ATP} + \mathrm{H_2O} \longrightarrow \mathrm{ADP} + \mathrm{P_i} \tag{4.2.1}$$

where ADP is adenosine diphosphate and $\mathrm{P_i}$ is inorganic phosphate.

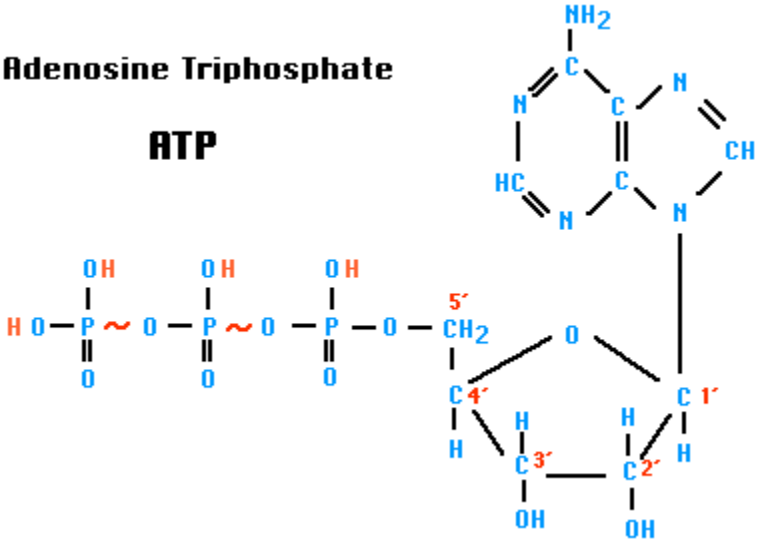


Figure 4.2.1: *ATP*

Because of the substantial amount of energy that is liberated when it is broken, the bond between the second and third phosphates is commonly described as a "high-energy" bond and is depicted in the figure by a wavy red line. (The bond between the first and second phosphates is also "high-energy".) (But please note that the term is **not** being used in the same sense as the term "bond energy". In fact, these bonds are actually weak bonds with low bond energies.)

Cells contain a wide variety of enzymes — called **ATPases** — that catalyze the hydrolysis of ATP and couple the energy released to particular energy-consuming reactions in the cell (see examples below).

Synthesis of ATP

- **ADP + Pi → ATP + H2O**
- requires energy: 7.3 kcal/mole
- occurs in the cytosol by **glycolysis**
- occurs in mitochondria by **cellular respiration**
- occurs in chloroplasts by **photosynthesis**

Consumption of ATP

ATP powers most of the energy-consuming activities of cells, such as:

- Most **anabolic reactions** such as
  - joining transfer RNAs to amino acids for assembly into proteins
  - synthesis of nucleoside triphosphates for assembly into DNA and RNA
  - synthesis of polysaccharides
  - synthesis of fats
- **active transport** of molecules and ions
- nerve impulses
- maintenance of cell volume by osmosis
- adding phosphate groups (**phosphorylation**) to many different proteins, e.g., to alter their activity in cell signaling
- muscle contraction
- beating of cilia and flagella (including sperm)
- bioluminescence

Extracellular ATP

In mammals, ATP also functions outside of cells. Its release

- from damaged cells can elicit inflammation and pain
- from the carotid body signals a shortage of oxygen in the blood
- from taste receptor cells triggers action potentials in the sensory nerves leading back to the brain
- from the stretched wall of the urinary bladder signals when the bladder needs emptying

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4.3: NAD and NADP

Nicotinamide adenine dinucleotide (NAD) and its relative nicotinamide adenine dinucleotide phosphate (NADP) are two of the most important coenzymes in the cell. NADP is simply NAD with a third phosphate group attached as shown at the bottom of the figure.

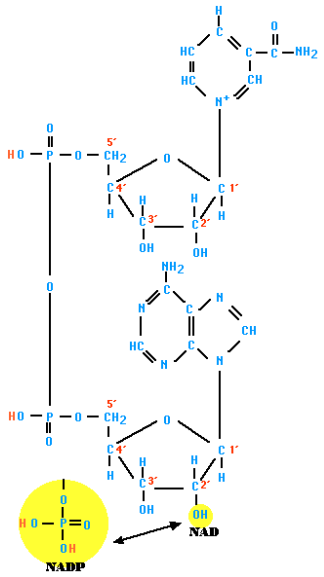


Figure 4.3.1: NAD and NADP

Because of the positive charge on the nitrogen atom in the nicotinamide ring (upper right), the oxidized forms of these important redox reagents are often depicted as NAD^+ and $NADP^+$ respectively.

In cells, most oxidations are accomplished by the removal of hydrogen atoms. Both of these coenzymes play crucial roles in this. Each molecule of NAD^+ (or $NADP^+$) can acquire two electrons; that is, be reduced by two electrons. However, only one proton accompanies the reduction. The other proton produced as two hydrogen atoms are removed from the molecule being oxidized is liberated into the surrounding medium. For NAD, the reaction is thus:

$$\mathrm{NAD^+ + 2\,H \longrightarrow NADH + H^+} \tag{4.3.1}$$

NAD and NADP uses

NAD participates in many redox reactions in cells, including those in glycolysis and most of those in the citric acid cycle of cellular respiration.

NADP is the reducing agent produced by the light reactions of photosynthesis and is consumed in the Calvin cycle of photosynthesis and used in many other anabolic reactions in both plants and animals.

Under the conditions existing in a normal cell, the hydrogen atoms shown in red are dissociated from these acidic substances.

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4.4: Glycolysis

Glycolysis is the anaerobic catabolism of glucose and occurs in virtually all cells (Figure 4.4.1). In eukaryotes, it occurs in the cytosol, where it converts a molecule of glucose into 2 molecules of pyruvic acid.

$$C_6H_{12}O_6 + 2NAD^+ \rightarrow 2C_3H_4O_3 + 2NADH + 2H^+ \tag{4.4.1}$$

The free energy stored in 2 molecules of pyruvic acid is somewhat less than that in the original glucose molecule;some of this difference is captured in 2 molecules of **ATP**.

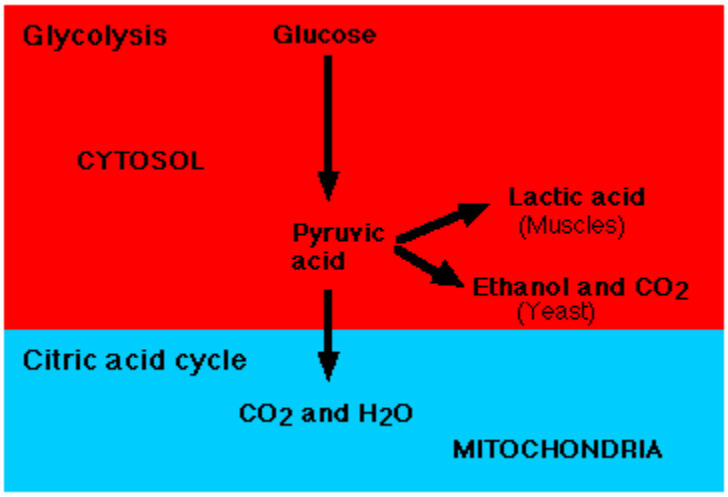


Figure 4.4.1: Glycolysis

The Fates of Pyruvic Acid

In **Yeasts,** Pyruvic acid is decarboxylated and reduced by NADH to form a molecule of carbon dioxide and one of ethanol.

$$C_3H_4O_3 + NADH + H^+ \rightarrow CO_2 + C_2H_5OH + NAD^+ \tag{4.4.2}$$

This accounts for the bubbles and alcohol in, for examples, beer and champagne via a process called alcoholic fermentation. The process is energetically wasteful because so much of the free energy of glucose (some 95%) remains in the alcohol (a good fuel).

In Red Blood Cells and active **Muscles,** Pyruvic acid is reduced by NADH forming a molecule of **lactic acid**.

$$C_3H_4O_3 + NADH + H^+ \rightarrow C_3H_6O_3 + NAD^+ \tag{4.4.3}$$

The process is called **lactic acid fermentation**. The process is energetically wasteful because so much free energy remains in the lactic acid molecule. (It can also be debilitating because of the drop in pH as the lactic acid produced in overworked muscles is transported out into the blood.)

In Mitochondria, Pyruvic acid is oxidized completely to form carbon dioxide and water via a process called **cellular respiration**. Approximately 40% of the energy in the original glucose molecule is trapped in molecules of **ATP**.

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4.5: Cellular Respiration

Cellular respiration is the process of oxidizing food molecules, like glucose, to carbon dioxide and water.

$$C_6H_{12}O_6 + 6O_2 + 6H_2O \rightarrow 12H_2O + 6CO_2 \tag{4.5.1}$$

The energy released is trapped in the form of **ATP** for use by all the energy-consuming activities of the cell. The process occurs in two phases:

- **glycolysis**, the breakdown of glucose to pyruvic acid
- the complete **oxidation of pyruvic acid** to carbon dioxide and water

In eukaryotes, glycolysis occurs in the cytosol and the remaining processes take place in **mitochondria**.

Mitochondria

Mitochondria are membrane-enclosed organelles distributed through the cytosol of most eukaryotic cells. Their number within the cell ranges from a few hundred to, in very active cells, thousands. Their main function is the conversion of the potential energy of food molecules into ATP.

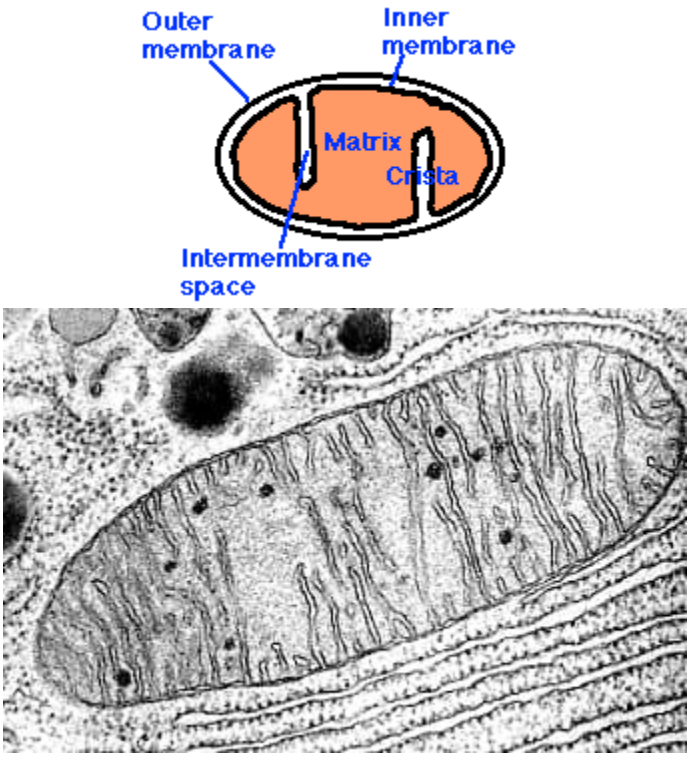


Figure 4.5.1: (left) Mitochondria (right) Mitochondrion from bat pancreas cell courtesy Keith R. Porter

Mitochondria have:

- an **outer membrane** that encloses the entire structure
- an **inner membrane** that encloses a fluid-filled **matrix**
- between the two is the **intermembrane space**
- the inner membrane is elaborately folded with shelflike **cristae** projecting into the **matrix**.
- a small number (some 5–10) circular molecules of **DNA**

This electron micrograph in Figure 4.5.1, shows a single mitochondrion from a bat pancreas cell. Note the double membrane and

the way the inner membrane is folded into cristae. The dark, membrane-bounded objects above the mitochondrion are lysosomes. The number of mitochondria in a cell can increase either by their fission (e.g. following mitosis) or decrease by their fusing together. Defects in either process can produce serious, even fatal, illness.

The Outer Membrane

The outer membrane contains many complexes of integral membrane proteins that form channels through which a variety of molecules and ions move in and out of the mitochondrion.

The Inner Membrane

The inner membrane contains 5 complexes of integral membrane proteins:

- **NADH dehydrogenase** (Complex I)
- succinate dehydrogenase (Complex II)
- **cytochrome c reductase** (Complex III; also known as the cytochrome b-c_1 complex)
- **cytochrome c oxidase** (Complex IV)
- **ATP synthase** (Complex V)

The Matrix

The matrix contains a complex mixture of soluble enzymes that catalyze the respiration of pyruvic acid and other small organic molecules. Here pyruvic acid is

- oxidized by NAD^+ producing NADH + H^+
- decarboxylated producing a molecule of
  - carbon dioxide (CO_2) and
  - a 2-carbon fragment of acetate bound to **coenzyme A** forming acetyl-CoA

The Citric Acid Cycle

This 2-carbon fragment is donated to a molecule of **oxaloacetic acid**. The resulting molecule of **citric acid** (which gives its name to the process) undergoes the series of enzymatic steps shown in the diagram. The final step regenerates a molecule of oxaloacetic acid and the cycle is ready to turn again.

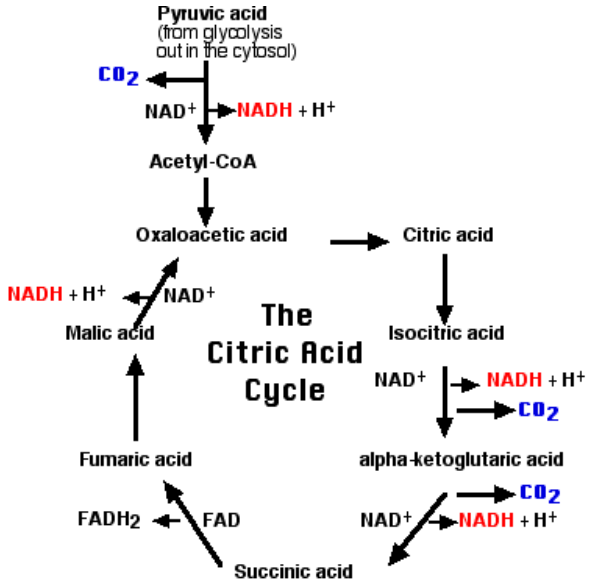


Figure 4.5.2: The Citric Acid Cycle

A brief summary of the cycle is as follows:

- Each of the 3 carbon atoms present in the pyruvate that entered the mitochondrion leaves as a molecule of carbon dioxide (**CO_2**).
- At 4 steps, a pair of electrons (**$2e^-$**) is removed and transferred to **NAD^+** reducing it to **NADH + H^+**.
- At one step, a pair of electrons is removed from succinic acid and reduces the prosthetic group flavin adenine dinucleotide (**FAD**) to **$FADH_2$**.
- The electrons of NADH and $FADH_2$ are transferred to the **electron transport chain**.

The Electron Transport Chain

The electron transport chain consists of 3 complexes of integral membrane proteins

- the **NADH dehydrogenase** complex (I)
- the **cytochrome c reductase** complex (III)
- the **cytochrome c oxidase** complex (IV)

and two freely-diffusible molecules **ubiquinone, cytochrome c,** that shuttle electrons from one complex to the next.

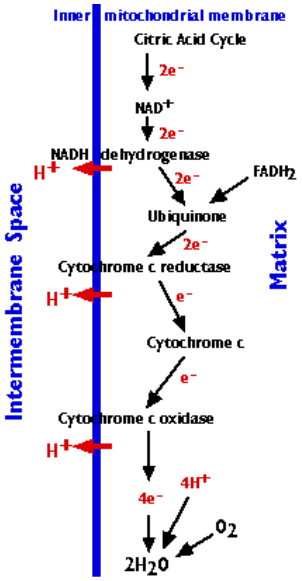


Figure 4.5.4 Electron Transport Chain

The electron transport chain accomplishes:

- The stepwise transfer of electrons from **NADH** (and **$FADH_2$**) to **oxygen** molecules to form (with the aid of protons) water molecules (**H_2O**). Cytochrome c can only transfer one electron at a time, so cytochrome c oxidase must wait until it has accumulated 4 of them before it can react with oxygen.
- Harnessing the energy released by this transfer to the pumping of protons (**H^+**) from the **matrix** to the **intermembrane space**.
- Approximately 20 protons are pumped into the intermembrane space as the 4 electrons needed to reduce oxygen to water pass through the respiratory chain.
- The gradient of protons formed across the inner membrane by this process of active transport forms a miniature battery.
- The protons can flow back down this gradient only by reentering the matrix through **ATP synthase**, another complex (complex V) of 16 integral membrane proteins in the inner membrane. The process is called chemiosmosis.

Chemiosmosis in mitochondria

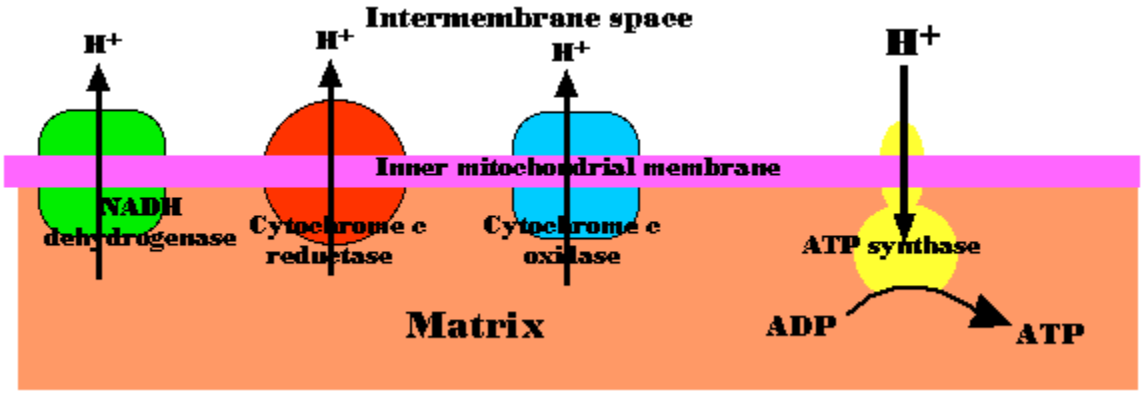


Fig.4.5.5 Chemiosmosis in Mitochondria

The energy released as electrons pass down the gradient from NADH to oxygen is harnessed by three enzyme complexes of the respiratory chain (I, III, and IV) to pump **protons** (H^+) **against** their concentration gradient **from the matrix** of the mitochondrion **into the intermembrane space.**

As their concentration increases there (which is the same as saying that the pH decreases), a strong diffusion gradient is set up. The only exit for these protons is through the **ATP synthase** complex. As in chloroplasts, the energy released as these protons flow down their gradient is harnessed to the synthesis of **ATP**. The process is called **chemiosmosis** and is an example of facilitated diffusion. One-half of the 1997 Nobel Prize in Chemistry was awarded to Paul D. Boyer and John E. Walker for their discovery of how ATP synthase works.

How many ATPs?

It is tempting to try to view the synthesis of ATP as a simple matter of stoichiometry (the fixed ratios of reactants to products in a chemical reaction). But (with 3 exceptions) **it is not**. Most of the ATP is generated by the proton gradient that develops across the inner mitochondrial membrane. The number of protons pumped out as electrons drop from NADH through the respiratory chain to oxygen is theoretically large enough to generate, as they return through ATP synthase, 3 ATPs per electron pair (but only 2 ATPs for each pair donated by $FADH_2$).

With 12 pairs of electrons removed from each glucose molecule,

- 10 by NAD^+ (so 10x3=30); and
- 2 by $FADH_2$ (so 2x2=4),

this could generate 34 ATPs. Add to this the 4 ATPs that are generated by the 3 exceptions and one arrives at 38. However, the energy stored in the proton gradient is also used for the active transport of several molecules and ions through the inner mitochondrial membrane into the matrix. NADH is also used as reducing agent for many cellular reactions. So the actual yield of ATP as mitochondria respire varies with conditions and probably seldom exceeds **30**.

The three exceptions

A stoichiometric production of ATP does occur at:

- one step in the citric acid cycle yielding 2 ATPs for each glucose molecule. This step is the conversion of alpha-ketoglutaric acid to succinic acid.
- at two steps in glycolysis yielding 2 ATPs for each glucose molecule.

Mitochondrial DNA (mtDNA)

The human mitochondrion contains 5–10 identical, circular molecules of DNA. Each consists of 16,569 base pairs carrying the information for **37 genes** which encode:

- 2 different molecules of **ribosomal RNA** (**rRNA**)
- 22 different molecules of **transfer RNA** (**tRNA**) (at least one for each amino acid)
- **13 polypeptides**

The **rRNA** and **tRNA** molecules are used in the machinery that synthesizes the 13 polypeptides.

The **13 polypeptides** participate in building several protein complexes embedded in the inner mitochondrial membrane.

- 7 subunits that make up the mitochondrial **NADH dehydrogenase** (complex I)
- **cytochrome b**, a subunit of cytochrome c reductase (complex III)
- 3 subunits of **cytochrome c oxidase** (complex IV)
- 2 subunits of **ATP synthase** (complex V)

Each of these protein complexes also requires subunits that are encoded by nuclear genes, synthesized in the cytosol, and imported from the cytosol into the mitochondrion. Nuclear genes also encode ~1,000 other proteins that must be imported into the mitochondrion.

Mutations in mtDNA cause human diseases

Mutations in 12 of the 13 polypeptide-encoding mitochondrial genes have been found to cause human disease. Although many different organs may be affected, disorders of the muscles and brain are the most common. Perhaps this reflects the great demand for energy of both these organs. (Although representing only ~2% of our body weight, the brain consumes ~20% of the energy produced when we are at rest.)

Some of these disorders are inherited in the germline. In every case, the mutant gene is received from the mother because none of the mitochondria in sperm survives in the fertilized egg. Other disorders are somatic; that is, the mutation occurs in the somatic tissues of the individual.

Example: Exercise Intolerance

A number of humans who suffer from easily-fatigued muscles turn out to have a mutations in their **cytochrome b** gene. Curiously, only the mitochondria in their muscles have the mutation; the mtDNA of their other tissues is normal. Presumably, very early in their embryonic development, a mutation occurred in a cytochrome b gene in the mitochondrion of a cell destined to produce their muscles.

The severity of mitochondrial diseases varies greatly. The reason for this is probably the extensive mixing of mutant DNA and normal DNA in the mitochondria as they fuse with one another. A mixture of both is called **heteroplasmy**. The higher the ratio of mutant to normal, the greater the severity of the disease. In fact by chance alone, cells can on occasion end up with all their mitochondria carrying all-mutant genomes — a condition called **homoplasmy** (a phenomenon resembling genetic drift).

Mitochondrial Replacement Techniques

As I noted above, only mothers can pass mutant mtDNA on to their offspring. Two techniques are under intense investigation, either of which could enable a mother to have children free of defective mitochondria. Mutations in some 228 nuclear genes have also been implicated in human mitochondrial diseases, but mitochondrial replacement techniques will not be able to help with these.

Why do mitochondria have their own genome?

Many of the features of the mitochondrial genetic system resemble those found in bacteria. This has strengthened the theory that

mitochondria are the evolutionary descendants of a bacterium that established an endosymbiotic relationship with the ancestors of eukaryotic cells early in the history of life on earth. However, many of the genes needed for mitochondrial function have since moved to the nuclear genome. The recent sequencing of the complete genome of *Rickettsia prowazekii* has revealed a number of genes closely related to those found in mitochondria. Perhaps rickettsias are the closest living descendants of the endosymbionts that became the mitochondria of eukaryotes.

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4.6: ATP Synthase

ATP synthase is a huge molecular complex (>500,000 daltons) embedded in the inner membrane of mitochondria. Its function is to convert the energy of protons (H^+) moving down their concentration gradient into the synthesis of ATP. 3 to 4 protons moving through this machine is enough to convert a molecule of ADP and P_i (inorganic phosphate) into a molecule of ATP. One ATP synthase complex can generate >100 molecules of ATP each second.

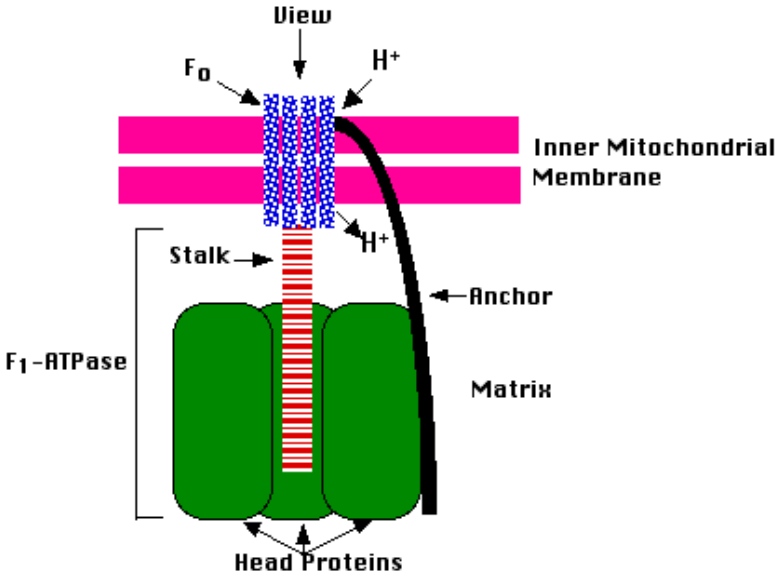


Figure 4.6.1: ATP Synthase

ATP synthase can be separated into 2 parts:

- F_O - the portion embedded in the inner mitochondrial membrane
- F_1-ATPase — the portion projecting into the matrix of the mitochondrion

This is why the intact ATP synthase is also called the F_OF_1-ATPase.

When the F_1-ATPase is isolated in vitro, it catalyzes the hydrolysis of ATP to ADP and P_i (which is why it is called the F_1-ATPase). While it is doing so, the central portion of F_O attached to the stalk rotates rapidly in a counter-clockwise direction (as viewed from above).

In the intact mitochondrion, the protons that have accumulated in the intermembrane space enter the F_O complex and exit from it into the matrix. The energy they give up as they travel down their concentration gradient rotates F_O and its stalk (at ~6000 rpm) in a clockwise direction. As it does so, it induces repeating conformational changes in the head proteins that enable them to convert ADP and P_i into ATP. (In the figure, two of the three dimers that make up the head proteins have been pulled aside to reveal the stalk inserted in their center.)

In both these cases, the machine is converting chemical energy from the hydrolysis of ATP in the in vitro case and the flow of protons down their concentration gradient in the intact mitochondrion into mechanical energy — the turning of the motor. But this remarkable device can be made to do the reverse, converting mechanical energy (turning of the motor) into chemical energy.

A group of Japanese scientists interested in nano-machines have succeeded in attaching magnetic beads to the stalks of the F_1-ATPase isolated *in vitro*. Then using a rotating magnetic field they were able to make the stalks rotate. When rotated in a clockwise direction, the F_1-ATPase synthesized ATP from ADP and P_i in the surrounding medium — at a rate of about 5 molecules per second! (When rotating the stalks in the counter-clockwise direction, or not rotating them at all, ATP was hydrolyzed into ADP and P_i.)

Their achievement was reported in Itoh, H., *et al*., **Nature**, 29 January 2004.

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4.7: Photosynthesis - Pathway of Carbon Fixation

Photosynthesis is the synthesis of organic molecules using the energy of light. For the sugar glucose (one of the most abundant products of photosynthesis) the equation is:

$$6\,CO_2 + 12\,H_2O \longrightarrow C_6H_{12}O_6 + 6\,H_2O + 6\,O_2 \tag{4.7.1}$$

Light provides the energy to transfer electrons from water to nicotinamide adenine dinucleotide phosphate ($NADP^+$) forming $NADPH$ and to generate ATP. Both ATP and NADPH provide the energy and electrons to reduce carbon dioxide (CO_2) to organic molecules.

The Steps that lead to Photosynthesis

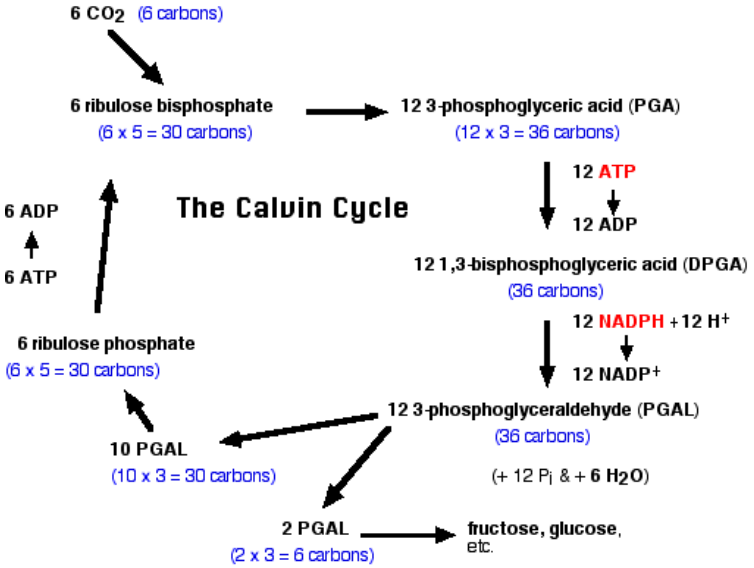


Figure 4.7.1: The steps in the fixation of carbon dioxide during photosynthesis

- CO_2 combines with the phosphorylated 5-carbon sugar **ribulose bisphosphate**.
- This reaction is catalyzed by the enzyme **ribulose bisphosphate carboxylase oxygenase** (**RUBISCO**)(an enzyme which can fairly claim to be the most abundant protein on earth).
- The resulting 6-carbon compound breaks down into two molecules of **3-phosphoglyceric acid** (**PGA**).
- The PGA molecules are further phosphorylated (by **ATP**) and are reduced (by **NADPH**) to form **phosphoglyceraldehyde** (**PGAL**).
- Phosphoglyceraldehyde serves as the starting material for the synthesis of **glucose** and **fructose**.
- Glucose and fructose make the disaccharide **sucrose**, which travels in solution to other parts of the plant (e.g., fruit, roots).
- Glucose is also the monomer used in the synthesis of the **polysaccharides starch** and **cellulose**.

The Figure 4.7.1 shows the steps in the fixation of carbon dioxide during photosynthesis. All of these reactions occur in the **stroma** of the chloroplast. These steps were worked out by Melvin Calvin and his colleagues at the University of California and, for this reason, are named the **Calvin cycle**.

Calvin's Experiment

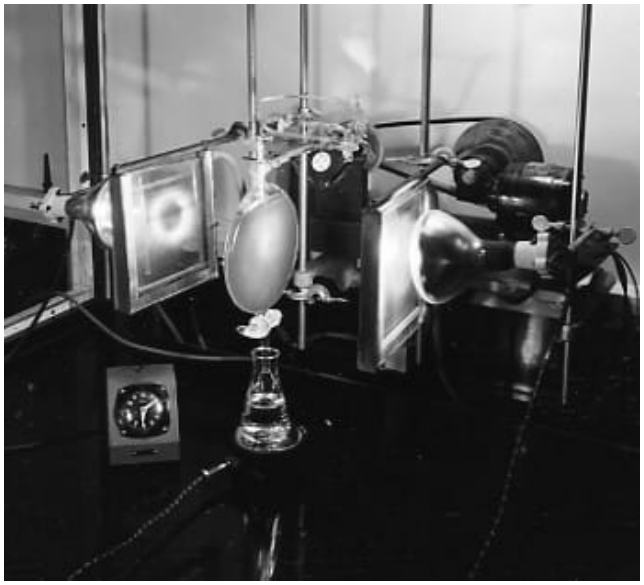


Figure 4.7.2 Apparatus used for Calvin's experiment courtesy of Dr. James A. Bassham

The experimental apparatus is shown above. After various intervals of illumination, a suspension of unicellular algae is inactivated and the contents of the cells extracted. The compounds in a drop of the extract are then separated by paper chromatography.

The identity of each substance may be determined simply by comparing its position with the positions occupied by known substances under the same conditions. Or, a fragment containing the spot can be cut from the sheet and chemically analyzed.

To determine which, if any, of the substances separated on the chromatogram are radioactive, a sheet of X-ray film is placed next to the chromatogram. If dark spots appear on the film (because of radiation emitted by the ^{14}C atoms), their position can be correlated with the positions of the chemicals in the chromatogram. Using this technique of **autoradiography**, Calvin found that ^{14}C turned up in glucose molecules within 30 seconds after the start of photosynthesis. When he permitted photosynthesis to proceed for only 5 seconds, however, the radioactivity was concentrated in several other, smaller, molecules.

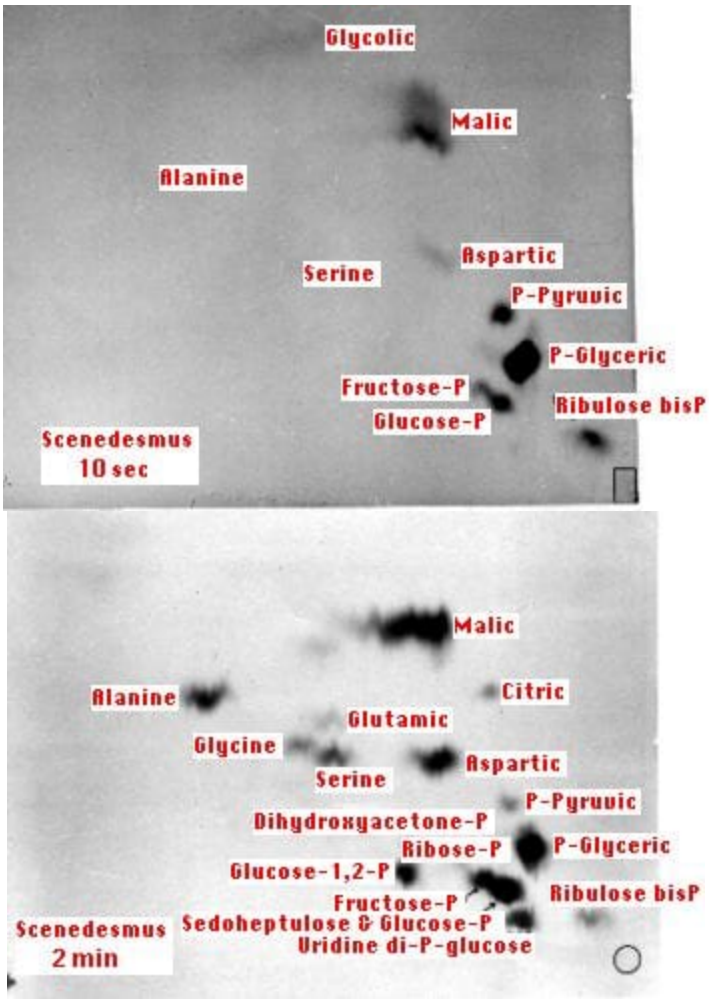


Figure 4.7.3 Chromatogram of Calvin's experiment courtesy of Dr. James A. Bassham

The dark spots show the radioactive compounds produced after 10 secs (left) and 2 minutes (right) of photosynthesis by the green alga Scenedesmus. The alga was supplied with carbon dioxide labeled with ^{14}C, a radioactive isotope of carbon. At 10 seconds, most of the radioactivity is found in 3-phosphoglyceric acid ("P-Glyceric"). At 2 minutes, phosphorylated 6-carbon sugars (glucose and fructose) have been synthesized as well as a number of amino acids. The small rectangle and circle (lower right-hand corners) mark the spots where the cell extract was applied.

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4.8: Photosynthesis - The Role of Light

The heart of photosynthesis as it occurs in most autotrophs consists of two key processes:

- **the removal of hydrogen (H) atoms from water molecules**
- **the reduction of carbon dioxide (CO_2) by these hydrogen atoms to form organic molecules**.

The second process involves a cyclic series of reactions named (after its discoverer) the **Calvin Cycle**.

The electrons (e^-) and protons (H^+) that make up hydrogen atoms are stripped away separately from water molecules.

$$2\,H_2O \longrightarrow 4\,e^- + 4\,H^+ + O_2 \tag{4.8.1}$$

The electrons serve two functions:

- They reduce $NADP^+$ to **NADPH** for use in the Calvin Cycle.
- They set up an electrochemical charge that provides the energy for pumping protons from the **stroma** of the **chloroplast** into the interior of the thylakoid.

The protons also serve two functions:

- They participate in the reduction of $NADP^+$ to NADPH.
- As they flow back out from the interior of the thylakoid (by facilitated diffusion), passing **down** their concentration gradient), the energy they give up is harnessed to the conversion of ADP to **ATP**.
- Because it is drive by light, this process is called **photophosphorylation**.

$$ADP + P_i \longrightarrow ATP \tag{4.8.2}$$

The ATP provides the second essential ingredient for running the Calvin Cycle.

The removal of electrons from water molecules and their transfer to $NADP^+$ requires energy. The electrons are moving from a redox potential of about +0.82 volt in water to −0.32 volt in NADPH. Thus enough energy must be available to move them against a total potential of 1.14 volts. Where does the needed energy come from? The answer: **Light**.

The Thylakoid Membrane

Chloroplasts contain a system of thylakoid membranes surrounded by a fluid **stroma**. Six different complexes of integral membrane proteins are embedded in the thylakoid membrane. The exact structure of these complexes differs from group to group (e.g., plant vs. alga) and even within a group (e.g., illuminated in air or underwater). They are as follows:

Photosystem I

The structure of photosystem I in a cyanobacterium ("blue-green alga") has been completely worked out. It probably closely resembles that of plants as well. It is a homotrimer with each subunit in the trimer containing:

- 12 different protein molecules bound to
- 96 molecules of **chlorophyll a**
  - 2 molecules of the **reaction center chlorophyll P700**
  - 4 accessory molecules closely associated with them
  - 90 molecules that serve as antenna pigments
- 22 **carotenoid** molecules
- 4 lipid molecules
- 3 clusters of Fe4S4

- 2 phylloquinones

Photosystem II

Photosystem II is also a complex of

- > 20 different protein molecules bound to
- 50 or more **chlorophyll a** molecules
  - 2 molecules of the **reaction center chlorophyll P680**
  - 2 accessory molecules close to them
  - 2 molecules of pheophytin (chlorophyll without the Mg^{++})
  - the remaining molecules of **chlorophyll a** serve as **antenna pigments**.
- some half dozen **carotenoid** molecules. These also serve as antenna pigments.
- 2 molecules of **plastoquinone**

Light-Harvesting Complexes (LHC)

- LHC-I associated with photosystem I
- LHC-II associated with photosystem II

These LHCs also act as **antenna pigments** harvesting light and passing its energy on to their respective photosystems.

The LHC-II of spinach is a homotrimer, with each monomer containing

- a single polypeptide
- 8 molecules of chlorophyll a
- 6 molecules of chlorophyll b
- 4 carotenoid molecules

Cytochromes b_6 and f

ATP synthase

How the System Works

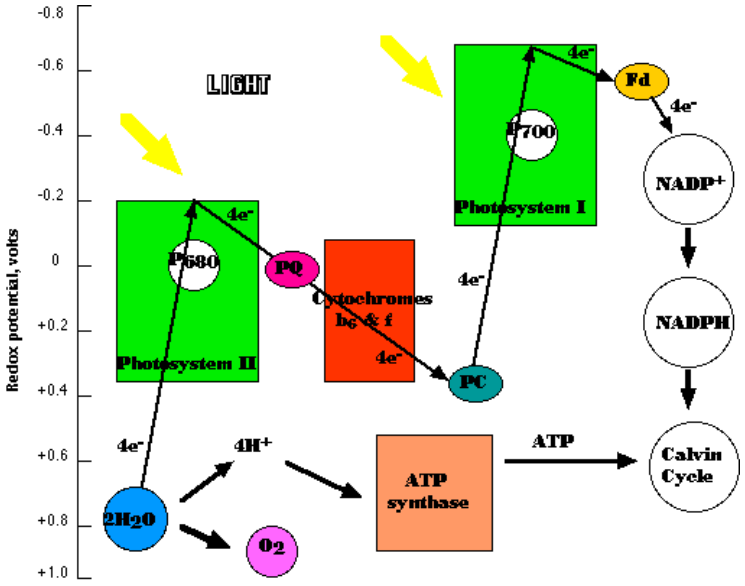


Figure 4.8.1: Photosystems

- Light is absorbed by the antenna pigments of **photosystems II** and **I**.
- The absorbed energy is transferred to the reaction center chlorophylls, **P680** in photosystem II, **P700** in photosystem I.
- Absorption of 1 photon of light by Photosystem **II** removes 1 electron from **P680**.
- With its resulting positive charge, P680 is sufficiently electronegative that it can remove 1 electron from a molecule of water.
- When these steps have occurred 4 times, requiring 2 molecules of water, 1 molecule of oxygen and 4 protons (H^+) are released
- The electrons are transferred (by way of **plastoquinone** — **PQ** in the figure) to the **cytochrome b6/f** complex where they provide the energy for **chemiosmosis**.
- Activation of **P700** in photosystem **I** enables it to pick up electrons from the cytochrome b6/f complex (by way of plastocyanin — **PC** in the figure) and raise them to a sufficiently high redox potential that, after passing through **ferredoxin** (**Fd** in the figure),
- they can reduce $NADP^+$ to **NADPH**.

The sawtooth shifts in redox potential as electrons pass from P680 to $NADP^+$ have caused this system to be called the **Z-Scheme** (although as I have drawn the diagram, it looks more like an "N"). It is also called **noncyclic photophosphorylation** because it produces ATP in a one-way process (unlike cyclic photophosphorylation and pseudocyclic photophosphorylation described below).

Chemiosmosis in Chloroplasts

The energy released as electrons pass down the gradient between photosystem II and plastocyanin (PC) is harnessed by the cytochrome b6/f complex to pump **protons** (H^+) **against** their concentration gradient from the stroma of the chloroplast into the interior of the thylakoid (an example of active transport). As their concentration increases inside (which is the same as saying that the pH of the interior decreases), a strong diffusion gradient is set up. The only exit for these protons is through the **ATP synthase** complex. As in mitochondria, the energy released as these protons flow down their gradient is harnessed to the synthesis of **ATP**. The process is called **chemiosmosis** and is an example of facilitated diffusion.

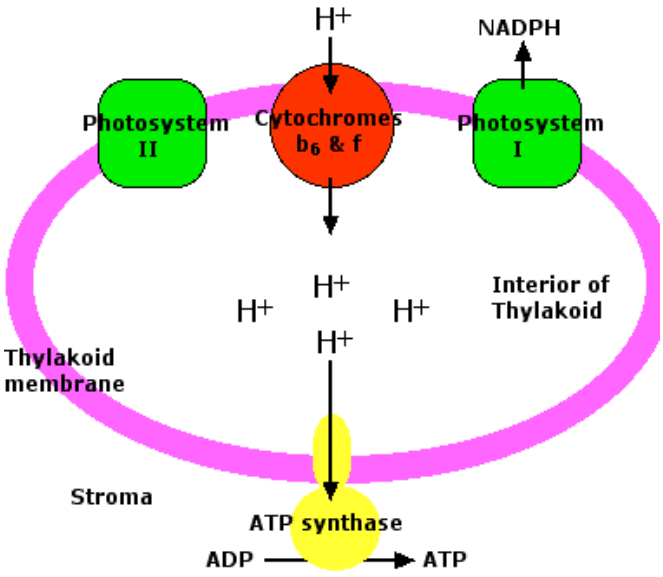


Figure 4.8.2: Chemiosmosis in Chloroplasts

Cyclic Photophosphorylation

- Each CO_2 taken up by the Calvin cycle) requires 2 NADPH molecules and 3 ATP molecules
- Each molecule of oxygen released by the light reactions supplies the 4 electrons needed to make 2 NADPH molecules.
- The chemiosmosis driven by these 4 electrons as they pass through the cytochrome b6/f complex liberates only enough energy to pump **12** protons into the interior of the thylakoid.

- But in order to make 3 molecules of ATP, the ATPase in chloroplasts appears to have **14** protons (H^+) pass through it.
- So there appears to be a deficit of 2 protons.
- How is this deficit to be made up?
- One likely answer: **cyclic photophosphorylation**.

In cyclic photophosphorylation,

- the electrons expelled by the energy of light absorbed by photosystem I pass, as normal, to ferredoxin (Fd).
- But instead of going on to make NADPH,
- they pass to plastoquinone (PQ) and on back into the cytochrome b_6/f complex.
- Here the energy each electron liberates pumps 2 protons (H^+) into the interior of the
- thylakoid — enough to make up the deficit left by noncyclic photophosphorylation.

This process is truly cyclic because no outside source of electrons is required. Like the photocell in a light meter, photosystem I is simply using light to create a flow of current. The only difference is that instead of using the current to move the needle on a light meter, the chloroplast uses the current to help synthesize ATP.

Pseudocyclic Photophosphorylation

Another way to make up the deficit is by a process called pseudocyclic photophosphorylation in which some of the electrons passing to ferredoxin then reduce molecular oxygen back to H_2O instead of reducing $NADP^+$ to NADPH.

At first glance, this might seem a fruitless undoing of all the hard work of photosynthesis. But look again. Although the electrons cycle from water to ferredoxin and back again, part of their pathway is through the chemiosmosis-generating stem of cytochrome b_6/f. Here, then, is another way that simply by turning on a light, enough energy is imparted to electrons that they can bring about the synthesis of ATP.

Antenna Pigments

Chlorophylls a and b differ slightly in the wavelengths of light that they absorb best (although both absorb red and blue much better than yellow and green). Carotenoids help fill in the gap by strongly absorbing green light. The entire complex ensures that most of the energy of light will be trapped and passed on to the reaction center chlorophylls.

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4.9: Photosynthesis - Dicovering the Secrets

This chapter talks about various scientists and their path towards discovering photosynthesis.

van Helmont

Perhaps the first experiment designed to explore the nature of photosynthesis was that reported by the Dutch physician van Helmont in 1648. Some years earlier, van Helmont had placed in a large pot exactly 200 pounds (91 kg) of soil that had been thoroughly dried in an oven. Then he moistened the soil with rain water and planted a 5-pound (2.3 kg) willow shoot in it. He then placed the pot in the ground and covered its rim with a perforated iron plate. The perforations allowed water and air to reach the soil but lessened the chance that dirt or other debris would be blown into the pot from the outside.

For five years, van Helmont kept his plant watered with rain water or distilled water. At the end of that time, he carefully removed the young tree and found that it had gained 164 pound, 3 ounces (74.5 kg). (This figure did not include the weight of the leaves that had been shed during the previous four autumns.) He then redried the soil and found that it weighed only 2 ounces (57 g) less that the original 200 pounds (91 kg). Faced with these experimental facts, van Helmont theorized that the increase in weight of the willow arose from the water alone. He did not consider the possibility that gases in the air might be involved.

Joseph Priestley

The first evidence that gases participate in photosynthesis was reported by Joseph Priestley in 1772. He knew that if a burning candle is placed in a sealed chamber, the candle soon goes out. If a mouse is then placed in the chamber, it soon suffocates because the process of combustion has used up all the oxygen in the air — the gas on which animal respiration depends. However, Priestley discovered that if a plant is placed in an atmosphere lacking oxygen, it soon replenishes the oxygen, and a mouse can survive in the resulting mixture. Priestley thought (erroneously) that it was simply the growth of the plant that accounted for this.

Ingen-Housz

It was another Dutch physician, Ingen-Housz, who discovered in 1778 that the effect observed by Priestley occurred only when the plant was illuminated. A plant kept in the dark in a sealed chamber consumes oxygen just as a mouse (or candle) does.

Ingen-Housz also demonstrated that only green parts of plants liberated oxygen during photosynthesis. Nongreen plant structure, such as woody stems, roots, flowers, and fruits actually consume oxygen in the process of respiration. We now know that this is because photosynthesis can go on only in the presence of the green pigment chlorophyll.

Jean Senebier

The growth of plants is accompanied by an increase in their carbon content. A Swiss minister, Jean Senebier, discovered that the source of this carbon is carbon dioxide and that the release of oxygen during photosynthesis accompanies the uptake of carbon dioxide. Senebier concluded (erroneously as it turned out) that in photosynthesis carbon dioxide is decomposed, with the carbon becoming incorporated in the organic matter of the plant and the oxygen being released.

$$CO_2 + H_2O \rightarrow (CH_2O) + O_2$$

(The parentheses around the CH_2O signify that no specific molecule is being indicated but, instead, the ratio of atoms in some carbohydrate, e.g., glucose, $C_6H_{12}O_6$.) The equation also indicates that the ratio of carbon dioxide consumed to oxygen release is 1:1, a finding that was carefully demonstrated in the years following Senebier's work. Using glucose as the carbohydrate product, we can write the equation for photosynthesis as

$$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$$

F. F. Blackman

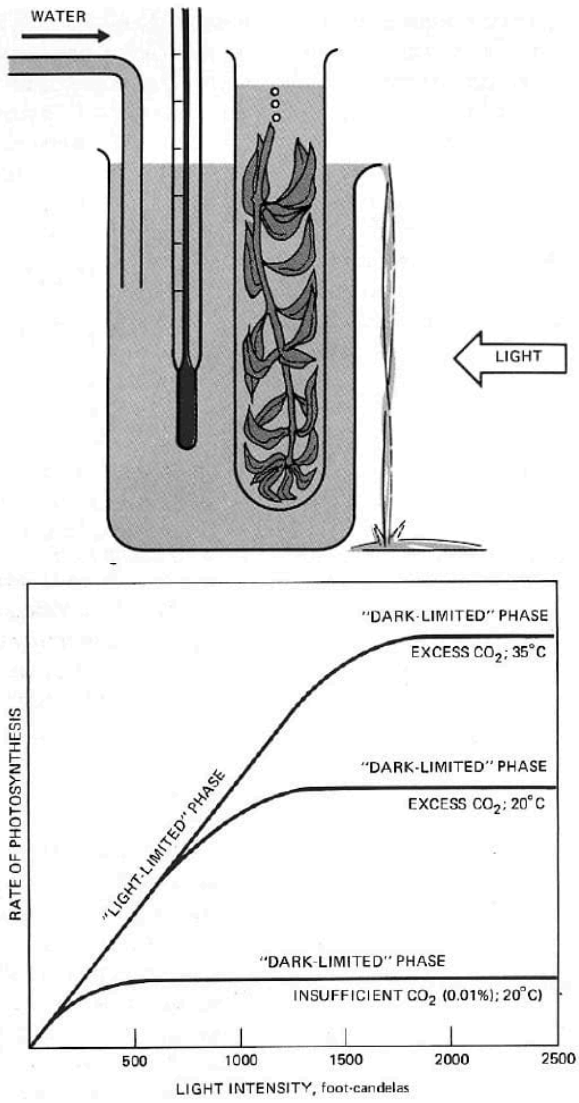


Figure 4.9.1: (left) Elodea experiment by Blackman (right) Blackman experiment graphic interpretation

The above equation shows the relationship between the substances used in and produced by the process. It tells us nothing about the intermediate steps. That photosynthesis does involve at least two quite distinct processes became apparent from the experiments of the British plant physiologist F. F. Blackman. His results can easily be duplicated by using the setup in Figure 4.9.1. The green water plant *Elodea* (available wherever aquarium supplies are sold) is the test organism. When a sprig is placed upside down in a dilute solution of $NaHCO_3$ (which serves as a source of CO_2) and illuminated with a flood lamp, oxygen bubbles are soon given off from the cut portion of the stem. One then counts the number of bubbles given off in a fixed interval of time at each of several light intensities. Plotting these data produces a graph like the one in Figure 4.9.2.

Since the rate of photosynthesis does not continue to increase indefinitely with increased illumination, Blackman concluded that at least two distinct processes are involved: one, a reaction that requires light and the other, a reaction that does not. This latter is called a "dark" reaction although it **can** go on in the light. Blackman theorized that at moderate light intensities, the "light" reaction limits or "paces" the entire process. In other words, at these intensities the dark reaction is capable of handling all the intermediate substances produced by the light reaction. With increasing light intensities, however, a point is eventually reached when the dark reaction is working at maximum capacity. Any further illumination is ineffective, and the process reaches a steady rate.

This interpretation is strengthened by repeating the experiment as a somewhat higher temperature. Most chemical reactions

proceed more rapidly at higher temperatures (up to a point). At 35°C, the rate of photosynthesis does not level off until greater light intensities are present. This suggest that the dark reaction is now working faster. The fact that at low light intensities the rate of photosynthesis is no greater at 35°C than at 20°C also supports the idea that it is a light reaction that is limiting the process in this range. Light reactions depend, not on temperature, but simply on the intensity of illumination.

The increased rate of photosynthesis with increased temperature does not occur if the supply of CO_2 is limited. As the figure shows, the overall rate of photosynthesis reaches a steady value at lower light intensities if the amount of CO_2 available is limited. Thus CO_2 concentration must be added as a third factor regulating the rate at which photosynthesis occurs. As a practical matter, however, the concentration available to terrestrial plants is simply that found in the atmosphere: 0.035%.

Van Niel

It was the American microbiologist Van Niel who first glimpsed the role that light plays in photosynthesis. He studied photosynthesis in purple sulfur bacteria. These microorganisms synthesize glucose from CO_2 as do green plants, and they need light to do so. Water, however, is not the starting material. Instead they use hydrogen sulfide (H_2S). Furthermore, no oxygen is liberated during this photosynthesis but rather elemental sulfur. Van Niel reasoned that the action of light caused a decomposition of H_2S into hydrogen and sulfur atoms. Then, in a series of dark reactions, the hydrogen atoms were used to reduce CO_2 to carbohydrate:

$$\mathrm{CO_2 + 2\,H_2S \longrightarrow (CH_2O) + H_2O + 2\,S} \tag{4.9.1}$$

Van Niel envisioned a parallel to the process of photosynthesis as it occurs in green plants. There the energy of light causes water to break up into hydrogen and oxygen. The hydrogen atoms are then used to reduce CO_2 in a series of dark reactions:

$$\mathrm{CO_2 + 2\,H_2O \longrightarrow (CH_2O) + H_2O + O_2} \tag{4.9.2}$$

If this theory is correct, then it follows that all of the oxygen released during photosynthesis comes from water just as all the sulfur produced by the purple sulfur bacteria comes from H_2S. This conclusion directly contradicts Senebier's theory that the oxygen liberated in photosynthesis comes from the carbon dioxide. If Van Niel's theory is correct, then the equation for photosynthesis would have to be rewritten:

$$\mathrm{6\,CO_2 + 12\,H_2O \longrightarrow C_6H_{12}O_6 + 6\,H_2O + 6\,O_2} \tag{4.9.3}$$

In science, a theory should be testable. By deduction, one can make a prediction of how a particular experiment will come out if the theory is sound. In this case, the crucial experiments needed to test the two theories had to await the time when the growth of atomic research made it possible to produce isotopes other than those found naturally or in greater concentrations than are found naturally.

Samuel Ruben

In air, water and other natural materials containing oxygen, 99.76% of the oxygen atoms are ^{16}O and only 0.20% of them are the heavier isotope ^{18}O. In 1941, Samuel Ruben and his coworkers at the University of California were able to prepare specially "labeled" water in which the 0.85% of the molecules contained ^{18}O atoms. When this water was supplied to a suspension of photosynthesizing algae, the proportion of ^{18}O in the oxygen gas that was evolved was 0.85%, the same as that of the water supplied, and not simply the 0.20% found in all natural samples of oxygen (and its compounds like CO_2).

		% ^{18}O FOUND IN		
EXPERIMENT		H_2O	CO_2	O_2
1.	START	0.85	0.20	—
	FINISH	0.85	0.61*	0.86
2.	START	0.20	0.68	—
	FINISH	0.20	0.57	0.20

* A non-biochemical exchange of oxygen atoms between the water and the bicarbonate ions used as a source of CO_2 explains the uptake of the isotope by CO_2 in the first experiment.

These results clearly demonstrated that Senebier's interpretation was in error. If all the oxygen liberated during photosynthesis comes from the carbon dioxide, we would expect the oxygen evolved in Ruben's experiment to contain simply the 0.20% found naturally. If, on the other hand, both the carbon dioxide and the water contribute to the oxygen released, we would expect its isotopic composition to have been some intermediate figure. In fact, the isotopic composition of the evolved oxygen was the same as that of the water used.

Ruben and his colleagues also prepared a source of carbon dioxide that was enriched in ^{18}O atoms. When algae carried out photosynthesis using this material and **natural** water, the oxygen that was given off was not enriched in ^{18}O. It contained simply the 0.20% ^{18}O found in the natural water used. The heavy atoms presumably became incorporated in the other two products (carbohydrate and by-product water).

These experiments lent great support to Van Niel's idea that one function of light in photosynthesis was the separation of the hydrogen and oxygen atoms of water molecules. But there remained to work out just how the hydrogen atoms were made available to the dark reactions.

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4.10: Chemiosmosis

Several kinds of evidence support the chemiosmotic theory of ATP synthesis in chloroplasts. When isolated chloroplasts are illuminated, the medium in which they are suspended becomes alkaline — as we would predict if protons were being removed from the medium and pumped into the thylakoids (where they reduce the pH to about 4.0 or so). The interior of thylakoids can be deliberately made acid (low pH) by suspending isolated chloroplasts in an acid medium (pH 4.0) for a period of time. When these chloroplasts are then transferred to a slightly alkaline medium (pH 8.5), that is, one with a lower concentration of protons and given a supply of ADP and inorganic phosphate (P_i), they spontaneously synthesize ATP. No light is needed.

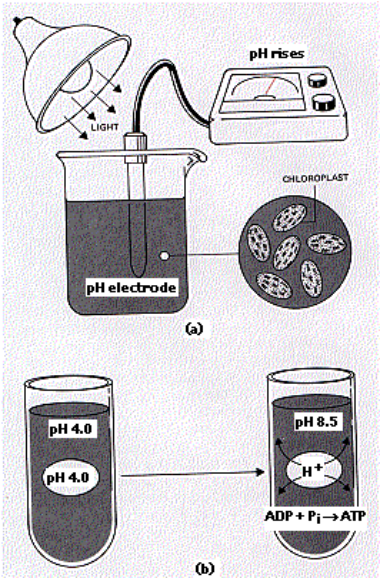


Figure 4.10.1: Chemiosmosis demo

This is a direct evidence that a gradient of protons can be harnessed to the synthesis of ATP.

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4.11: Metabolism

All living things must have an unceasing supply of energy and matter. The transformation of this energy and matter within the body is called **metabolism**.

Catabolism and Anabolism

Catabolism is **destructive metabolism**. Typically, in catabolism, larger organic molecules are broken down into smaller constituents. This usually occurs with the release of energy (usually as ATP). Anabolism is **constructive metabolism**. Typically, in anabolism, small precursor molecules are assembled into larger organic molecules. This always requires the input of energy (often as ATP).

Autotrophic vs. Heterotrophic Nutrition

Green plants, algae, and some bacteria are **autotrophs** ("self-feeders"). Most of them use the energy of sunlight to assemble inorganic precursors, chiefly carbon dioxide and water, into the array of organic macromolecules of which they are made. The process is **photosynthesis**. Photosynthesis makes the ATP needed for the anabolic reactions in the cell. All other organisms, including ourselves, are **heterotrophs**. We secure all our energy from organic molecules taken in from our surroundings ("food"). Although heterotrophs may feed partially (as most of us do) or exclusively on other heterotrophs, all the food molecules come ultimately from autotrophs. We may eat beef but the steer ate grass. Heterotrophs degrade some of the organic molecules they take in (catabolism) to make the ATP that they need to synthesize the others into the macromolecules of which they are made (anabolism).

How humans (and other animals) do it

Humans are heterotrophs. We are totally dependent on ingested preformed organic molecules to meet all our **energy** needs. We are also dependent on preformed organic molecules as the building blocks to meet our **anabolic** needs.

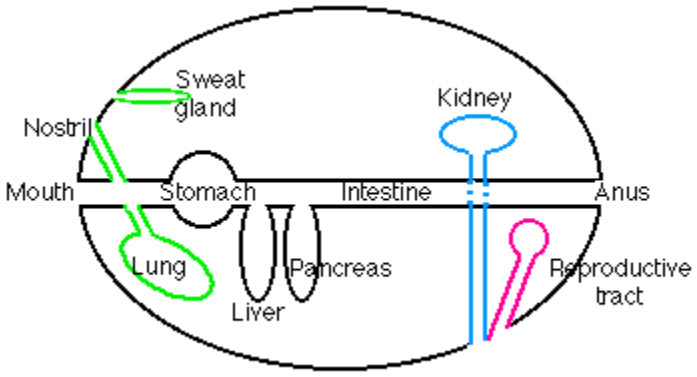


Figure 4.11.1: Human Topology

The steps for converting food to energy in animals:

1. **Ingestion**: taking food within the body (although as the figure shows, it is still topologically in the external world, not the internal).
2. **Digestion:** The enzyme-catalyzed hydrolysis of polysaccharides (e.g., starch) to sugars, proteins to amino acids, fats to fatty acids and glycerol, and nucleic acids to nucleotides.
3. **Absorption** into the body and transport to the cells.
4. **Absorption** into cells.

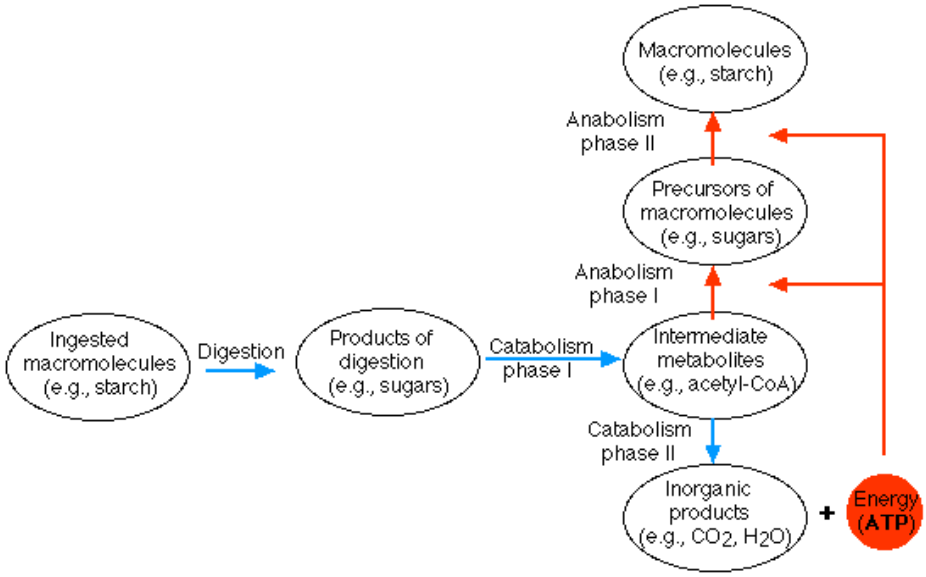


Figure 4.11.2: Metabolic Pathways

Within cells, these molecules are further degraded into still simpler molecules containing two to four carbon atoms. These fragments (**acetyl-CoA** for example) face one of two alternatives. They may proceed up various metabolic pathways and serve as the building blocks of, for example, sugars and fatty acids. From these will be assembled the macromolecules of the cell (e.g., polysaccharides, fats, proteins, and nucleic acids). Alternatively, the molecules in this pool of two- to four-carbon fragments may be still further degraded — ultimately to simple inorganic molecules such as carbon dioxide (CO_2), H_2O, and ammonia (NH_3). This phase of catabolism releases large amounts of energy (in the form of **ATP**). One use to which this energy is put is to run the anabolic activities of the cell.

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4.12: Intermediary Metabolism

The immediate source of energy for most cells is **glucose**. But glucose is not the only fuel on which cells depend. Other carbohydrates, fats and proteins may in certain cells or at certain times be used as a source of ATP. The complexity of the mechanism by which cells use glucose may make you fervently hope that a similarly-constructed system is not needed for each kind of fuel. And indeed it is not.

One of the great advantages of the step-by-step oxidation of glucose into CO_2 and H_2O is that several of the intermediate compounds formed in the process link glucose metabolism to the metabolism of other food molecules.

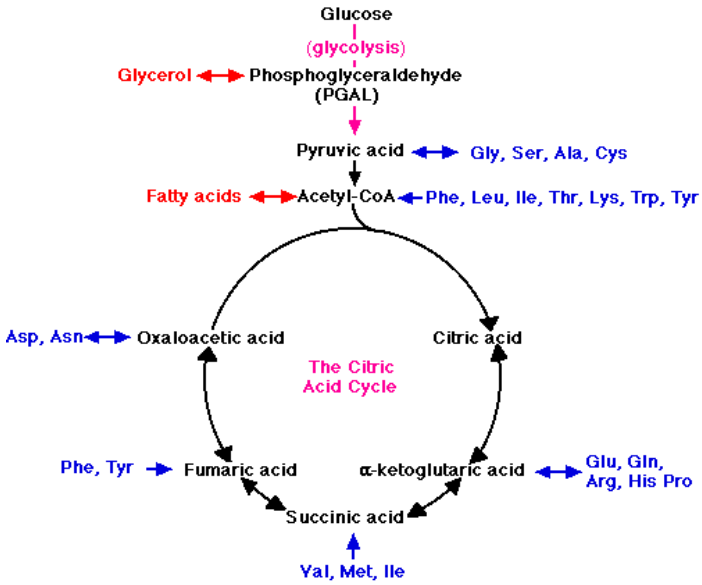


Figure 4.12.1: Intermediary Metabolism

For example, when fats are used as fuel, the glycerol portion of the molecule is converted into **PGAL** and enters the glycolytic pathway at that point. Fatty acids are converted into molecules of **acetyl-CoA** and enter the respiratory pathway to be oxidized in the mitochondria. The amino acids liberated by the hydrolysis of proteins can also serve as fuel.

- First, the nitrogen is removed, a process called deamination.
- The remaining fragments then enter the respiratory pathway at several points.

For examples,

- the amino acids Gly, Ser, Ala, and Cys are converted into pyruvic acid and enter the mitochondria to be respired.
- acetyl-CoA and several intermediates in the citric acid cycle serve as entry points for other amino acid fragments (shown in blue).

These links thus permit the respiration of excess fats and proteins in the diet. No special mechanism of cellular respiration is needed by those animals that depend largely on ingested fats (e.g., many birds) or proteins (e.g., carnivores) for their energy supply.

Much of the protein we consume is ultimately converted into glucose (a process called gluconeogenesis) to provide fuel for the brain and other tissues. Although all our foods are interconvertible to some extent, they are not completely so. In other words, no single food can supply all our anabolic needs. We can indeed synthesize many fats from glucose, but certain unsaturated fats cannot be synthesized and must be taken in directly in our diet. These are linoleic acid, linolenic acid, and arachidonic acid. All are unsaturated; that is, have double bonds. Although we can synthesize 11 of the amino acids from carbohydrate precursors, we must obtain 9 others (the "essential amino acids") directly.

Many of the points that connect carbohydrate metabolism to the catabolism of fats and proteins serve as two-way valves

(indicated in the figure by double-headed arrows). They provide points of entry not only for the catabolism (cellular respiration) of fatty acids, glycerol, and amino acids, but for their synthesis (anabolism) as well. Thus the catabolic breakdown of starches can lead (through acetyl-CoA and PGAL) to the synthesis of fat.

Fructose presents a special problem

Fructose is produced by the digestion of the disaccharide sucrose (common table sugar) into the monosaccharides glucose and fructose. Both glucose and fructose share the same empirical formula ($C_6H_{12}O_6$) and the same caloric content (686 kcal/mole). But the body treats them very differently.

Glucose is taken up and metabolized by all cells to generate ATP by glycolysis and cellular respiration, and excess glucose is preferentially converted into glycogen rather than fats. Fructose is taken up only by liver cells, and excess fructose is converted in fats (fatty acids and glycerol). In the U.S., most soft drinks and prepared foods are now sweetened with high-fructose (60% fructose, 40% glucose) corn syrup. Excessive consumption of these products may well be linked to the growing prevalence in the U.S. of obesity and type 2 diabetes.

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4.13: G Proteins

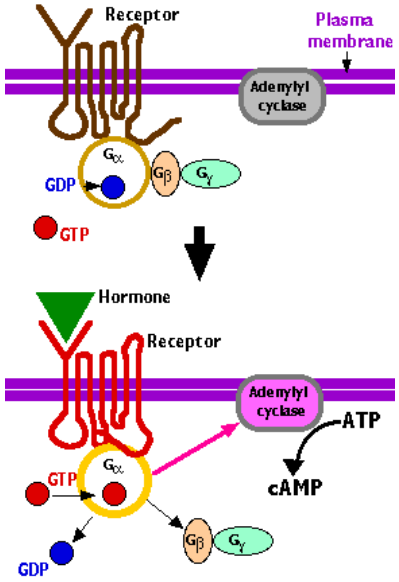


Figure 4.13.1: G Proteins

G proteins are so-called because they bind the **g**uanine nucleotides **G**DP and **G**TP. They are heterotrimers (i.e., made of three different subunits) associated with the inner surface of the plasma membrane and transmembrane **receptors** of hormones, etc. These are called G protein-coupled receptors (**GPCR**s).

The three subunits are:

- Gα, which carries the binding site for the nucleotide. At least 20 different kinds of Gα molecules are found in mammalian cells.
- Gβ
- Gγ

How G Proteins Work

- In the inactive state, Gα has **GDP** in its binding site.
- When a hormone or other ligand binds to the associated GPCR, an allosteric change takes place in the receptor (that is, its tertiary structure changes).
- This triggers an allosteric change in Gα causing
- GDP to leave and be replaced by **GTP**.
- GTP activates Gα causing it to dissociate from GβGγ (which remain linked as a dimer).
- Activated Gα in turn activates an effector molecule.

  In a common example (shown here), the effector molecule is **adenylyl cyclase** - an enzyme in the inner face of the plasma membrane which catalyzes the conversion of ATP into the "second messenger" cyclic AMP (**cAMP**).

Activated Gα is a GTPase so it quickly converts its GTP to GDP. This conversion, coupled with the return of the Gβ and Gγ subunits, restores the G protein to its inactive state.

Some Types of Gα Subunits

$G\alpha_s$

This type **stimulates** (**s** = "stimulatory") adenylyl cyclase. It is the one depicted here. It is associated with the receptors for many hormones such as:

- adrenaline
- glucagon
- luteinizing hormone (**LH**)
- parathyroid hormone (**PTH**)
- adrenocorticotropic hormone (**ACTH**)

$G\alpha_s$ is the target of the toxin liberated by Vibrio cholerae, the bacterium that causes **cholera**. Binding of cholera toxin to $G\alpha_s$ keeps it turned "on". The resulting continuous high levels of cAMP causes a massive loss of salts from the cells of the intestinal epithelium. Massive amounts of water follow by osmosis causing a diarrhea that can be fatal if the salts and water are not quickly replaced.

$G\alpha_q$

This activates **phospholipase C** (PLC) which generates the second messengers:

- inositol trisphosphate (**IP_3**)
- diacylglycerol (**DAG**)

$G\alpha_q$ is found in G proteins coupled to receptors for vasopressin, thyroid-stimulating hormone (**TSH**), and angiotensin.

$G\alpha_i$

This **inhibits** (**i** = "inhibitory") adenylyl cyclase lowering the level of cAMP in the cell. $G\alpha_i$ is activated by the receptor for somatostatin.

$G\alpha_t$

The "t" is for **transducin**, the molecule responsible for generating a signal in the rods of the retina in response to light. $G\alpha_t$ triggers the breakdown of **cyclic GMP** (cGMP).

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4.14: Secondary Messengers

Second messengers are molecules that relay signals received at receptors on the cell surface — such as the arrival of protein hormones, growth factors, etc. — to target molecules in the cytosol and/or nucleus. But in addition to their job as relay molecules, second messengers serve to greatly **amplify the strength** of the signal. Binding of a ligand to a single receptor at the cell surface may end up causing massive changes in the biochemical activities within the cell.

There are 3 major classes of second messengers:

1. cyclic nucleotides (e.g., **cAMP** and **cGMP**)
2. inositol trisphosphate (**IP3**) and diacylglycerol (**DAG**)
3. calcium ions (Ca^{2+})

Cyclic Nucleotides

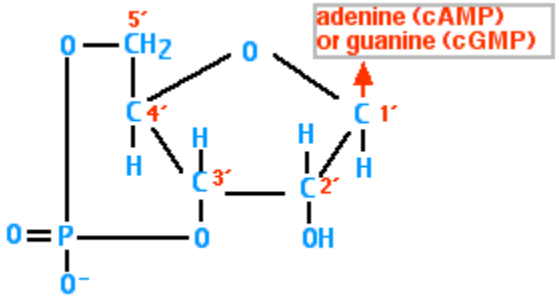


Figure 4.14.1: Cyclic Nucleotides

Cyclic AMP (cAMP)

Some of the hormones that achieve their effects through cAMP as a second messenger:

- adrenaline
- glucagon
- luteinizing hormone (LH)

Cyclic AMP is synthesized from ATP by the action of the enzyme **adenylyl cyclase**.

- Binding of the hormone to its receptor activates
- a **G protein** which, in turn, activates
- adenylyl cyclase.
- The resulting rise in cAMP turns on the appropriate response in the cell by either (or both):
  - changing the molecular activities in the cytosol, often using **P**rotein **K**inase **A** (**PKA**) — a c**A**MP-dependent protein kinase that phosphorylates target proteins
  - turning on a new pattern of gene transcription

Cyclic GMP (cGMP)

Cyclic GMP is synthesized from the nucleotide GTP using the enzyme **guanylyl cyclase**. Cyclic GMP serves as the second messenger for

- atrial natriuretic peptide (ANP)
- nitric oxide (NO)

- the response of the rods of the retina to light

Some of the effects of cGMP are mediated through **P**rotein **K**inase **G** (**PKG**) — a c**G**MP-dependent protein kinase that phosphorylates target proteins in the cell.

Inositol trisphosphate (IP3) and diacylglycerol (DAG)

Peptide and protein hormones like vasopressin, thyroid-stimulating hormone (**TSH**), and angiotensin and neurotransmitters like GABA bind to G protein-coupled receptors (GPCRs) that activate the intracellular enzyme **phospholipase C** (**PLC**).

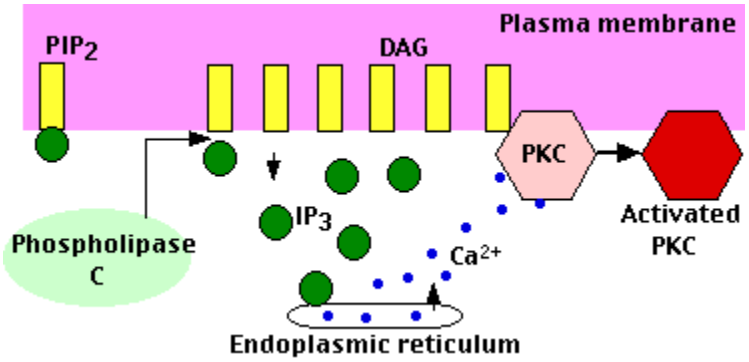


Figure 4.14.3 IP3 and DAG

As its name suggests, it hydrolyzes phospholipids — specifically phosphatidylinositol-4,5-bisphosphate (PIP_2) which is found in the inner layer of the plasma membrane. Hydrolysis of PIP_2 yields two products:

- **diacylglycerol** (**DAG**): DAG remains in the inner layer of the plasma membrane. It recruits **P**rotein **K**inase **C** (**PKC**) — a **c**alcium-dependent kinase that phosphorylates many other proteins that bring about the changes in the cell. As its name suggests, activation of PKC requires calcium ions. These are made available by the action of the other second messenger — IP_3.
- **inositol-1,4,5-trisphosphate** (**IP_3**): This soluble molecule diffuses through the cytosol and binds to receptors on the endoplasmic reticulum causing the release of calcium ions (Ca^{2+}) into the cytosol. The rise in intracellular calcium triggers the response.

Example:

The calcium rise is needed for NF-AT (the "nuclear factor of activated T cells") to turn on the appropriate genes in the nucleus.

The remarkable ability of **tacrolimus** and **cyclosporine** to prevent **graft rejection** is due to their blocking this pathway.

The binding of an antigen to its receptor on a B cell (the BCR) also generates the second messengers DAG and IP_3.

Calcium ions (Ca^{2+})

As the functions of IP_3 and DAG indicate, calcium ions are also important intracellular messengers. In fact, calcium ions are probably the most widely used intracellular messengers.

In response to many different signals, a rise in the concentration of Ca^{2+} in the cytosol triggers many types of events such as

- muscle contraction
- exocytosis, e.g.
  - release of neurotransmitters at synapses (and essential for the long-term synaptic changes that produce Long-Term Potentiation (LTP) and Long-Term Depression (LTD);
  - secretion of hormones like insulin
- activation of T cells and B cells when they bind antigen with their antigen receptors (TCRs and BCRs respectively)

- adhesion of cells to the extracellular matrix (ECM)
- apoptosis
- a variety of biochemical changes mediated by **P**rotein **K**inase **C** (**PKC**).

Normally, the level of calcium in the cell is very low (~100 nM). There are two main depots of Ca^{2+} for the cell:

- The extracellular fluid (ECF — made from blood), where the concentration is ~ 2 mM or 20,000 times higher than in the cytosol;
- the endoplasmic reticulum ("sarcoplasmic" reticulum in skeletal muscle).

However, its level in the cell can rise dramatically when channels in the plasma membrane open to allow it in from the extracellular fluid or from depots within the cell such as the endoplasmic reticulum and mitochondria.

Getting Ca^{2+} into (and out of) the cytosol

- Voltage-gated channels
  - open in response to a change in membrane potential, e.g. the depolarization of an action potential
  - are found in excitable cells:
    - skeletal muscle
    - smooth muscle (These are the channels blocked by drugs, such as felodipine [Plendil®], used to treat high blood pressure. The influx of Ca^{2+} contracts the smooth muscle walls of the arterioles, raising blood pressure. The drugs block this.)
    - neurons. When the action potential reaches the presynaptic terminal, the influx of Ca^{2+} triggers the release of the neurotransmitter.
    - the taste cells that respond to salt.
  - allow some 10^6 ions to flow in each second following the steep concentration gradient.
- Receptor-operated channels
  These are found in the post-synaptic membrane and open when they bind the neurotransmitter. Example: NMDA receptors.
- G-protein-coupled receptors (GPCRs). These are not channels but they trigger a release of Ca^{2+} from the endoplasmic reticulum as described above. They are activated by various hormones and neurotransmitters (as well as bitter substances on taste cells in the tongue).

Ca^{2+} ions are returned

- to the ECF by active transport using
  - an ATP-driven pump called a Ca^{2+} ATPase;
  - two Na^+/Ca^{2+} exchangers. These antiport pumps harness the energy of
    - 3 Na^+ ions flowing DOWN their concentration gradient to pump one Ca^{2+} against its gradient and
    - 4 Na^+ ions flowing down to pump 1 Ca^{2+} and 1 K^+ ion up their concentration gradients.
- to the endoplasmic (and sarcoplasmic) reticulum using another Ca^{2+} ATPase.

How can such a simple ion like Ca^{2+} regulate so many different processes? Some factors at work:

- localization within the cell (e.g., released at one spot — the T-system is an example — or spread throughout the cell)
- by the amount released (amplitude modulation, "AM")
- by releasing it in pulses of different frequencies (frequency modulation, "FM")

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4.15: Bioluminescence

Bioluminescence is the ability of living things to emit light. It is found in many marine animals, both invertebrate (e.g., some cnidarians, crustaceans, squid) and vertebrate (some fishes); some terrestrial animals (e.g., fireflies, some centipedes); and some fungi and bacteria. The molecular details vary from organism to organism, but each involves a **luciferin (**a light-emitting substrate), a **luciferase (**an enzyme that catalyzes the reaction), ATP (the source of energy) and molecular oxygen, O_2.

The more ATP available, the brighter the light. In fact, firefly luciferin and luciferase are commercially available for measuring the amount of ATP in biological materials. Fireflies use their flashes to attract mates. The pattern differs from species to species. In one species, the females sometimes mimic the pattern used by females of another species. When the males of the second species respond to these "femmes fatales", they are eaten!

How Fireflies Control their Flashing

Barry Trimmer and his colleagues at Tufts University have recently discovered how fireflies turn their luminescent organs (called **lanterns) ON**.



Figure 4.15.1: Firefly (species unknown) captured in eastern Canada – the top picture is taken with a flash, the bottom only with the self-emitted light. (CC SABy 3.0; Emmanuelm).

- The luminescent cells of the lanterns are close to cells at the end of the **tracheoles** (that bring oxygen to — and take carbon dioxide away from — the insect's tissues).
- These cells contain **nitric oxide synthase** (NOS), the enzyme that liberates the gas nitric oxide (**NO**) from arginine.
- Nerve impulses activate the release of NO from these cells.
- The NO diffuses into the lantern cells and inhibits cellular respiration in the mitochondria (probably by blocking the action of cytochrome c oxidase)
- With cellular respiration inhibited, the oxygen content of the cells increases.
- This turns on light production in the peroxisomes that contain luciferase and luciferin-ATP (the ATP is generated when the lanterns are dark).
- The quick decay of NO probably contributes to the short duration of the flash.

Bioluminescence in Marine Animals

The widespread occurrence of luminescence among deep-sea animals reflects the perpetual darkness in which they live. At least one fish has its luminescent organ located at the tip of a protruding stalk and uses it as bait to lure prey within reach of its jaws. When disturbed, one species of squid emits a cloud of luminescent water instead of the ink that its shallow-water relatives use. Some marine animals that live near the surface have luminescent organs on their underside. These probably make it more difficult for predators beneath them to see them against the light background of the surface.

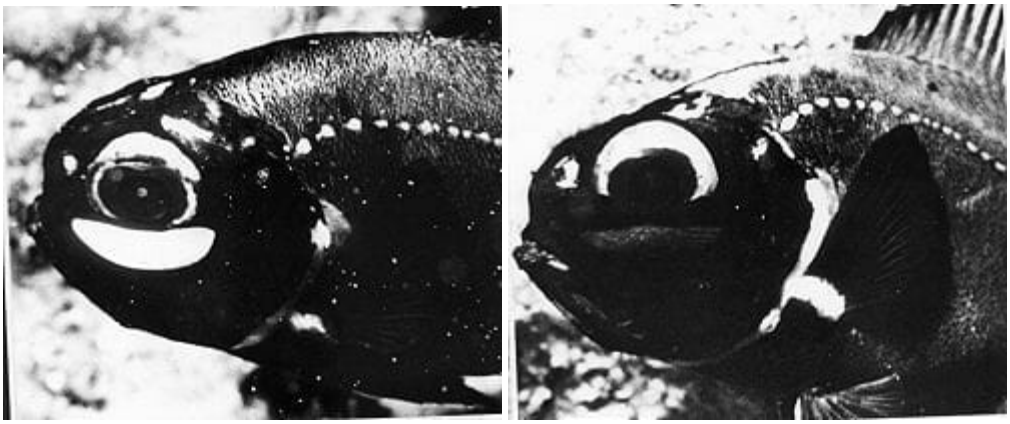


Fig 4.15.2: Bioluminescence in fish courtesy of Prof. J. W. Hastings

In the case of fishes, the light is emitted by luminescent bacteria that grow in luminescent organs. The photos show the flashlight fish, ***Photoblepharon palpebratus***, with the lid of its luminescent organ open (left) and closed (right). The light is produced by continuously-emitting luminescent bacteria within the organs, but its display is controlled by the fish. These animals, which were photographed along reefs in the Gulf of Elat, Israel, appear to use their luminescent organs for such varied functions as: (1) attracting prey, (2) signaling other members of their species, and (3) confusing potential predators.

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UNIT 5: DNA

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5.1: Transformation in Bacteria

Bacteria have no sexual reproduction in the sense that eukaryotes do. The have no alternation of diploid and haploid generations, no gametes, and no meiosis. However, the essence of sex is genetic recombination, and bacteria do have three mechanisms to accomplish that: transformation, conjugation and transduction.

Transformation

Many bacteria can acquire new genes by taking up DNA molecules (e.g., a plasmid) from their surroundings. The ability to deliberately transform the bacterium ***E. coli*** has made possible the cloning of many genes, including human genes, and the development of the biotechnology industry. The first demonstration of bacterial transformation was done with *Streptococcus pneumoniae* and led to the discovery that DNA is the substance of the genes. The path leading to this epoch-making discovery began in 1928 with the work of an English bacteriologist, Fred Griffith.

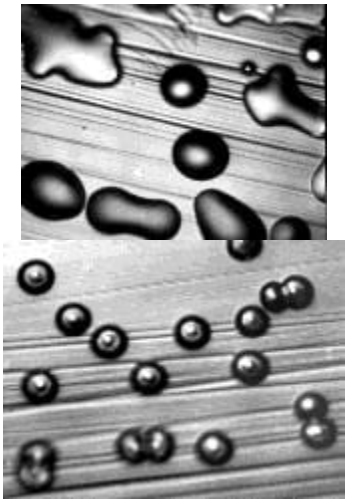


Figure 5.1.1: Streptococcus pneumoniae colonies. (left) Smooth (S) colonies and (right) Rough (R) colonies. Courtesy of Robert Austrian, J. Exp. Med. 98:21, 1953.

The cells of *S. pneumoniae* (also known as the pneumococcus) are usually surrounded by a gummy capsule made of a polysaccharide. When grown on the surface of a solid culture medium, the capsule causes the colonies to have a glistening, smooth appearance. These cells are called **"S"** cells. However, after prolonged cultivation on artificial medium, some cells lose the ability to form the capsule, and the surface of their colonies is wrinkled and rough (**"R"**). With the loss of their capsule, the bacteria also **lose their virulence**. Injection of a single **S** pneumococcus into a mouse will kill the mouse in 24 hours or so. But an injection of over 100 million (100×10^6) **R** cells is entirely harmless.

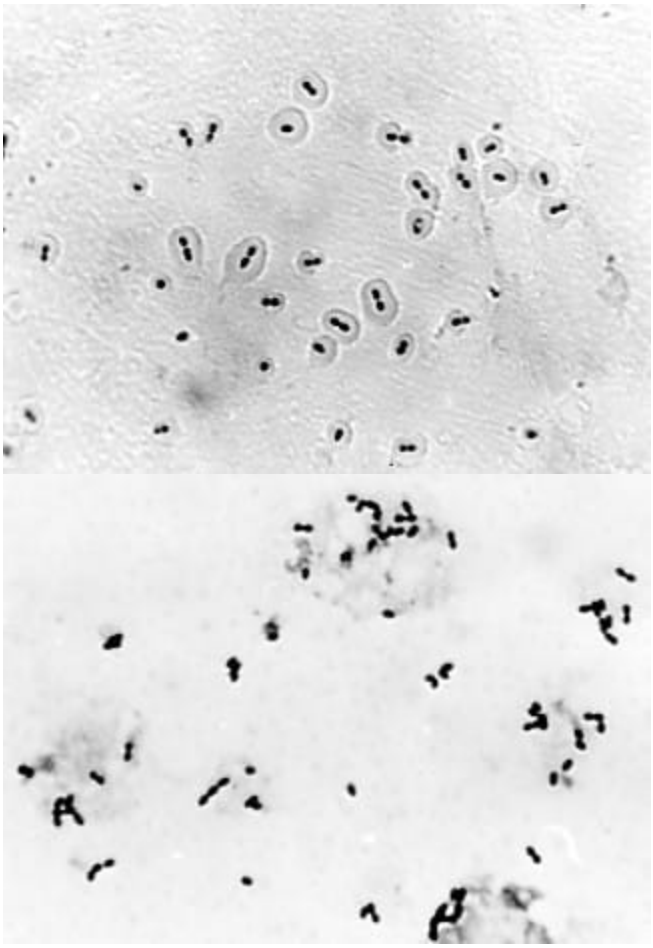


Figure 5.1.2: Encapsulated (left) and nonencapsulated (right) pneumococci Courtesy of Robert Austrian, J. Exp. Med. 98:21, 1953

The reason? The capsule prevents the pneumococci from being engulfed and destroyed by scavenging cells, neutrophils and macrophages, in the body. The R forms are completely at the mercy of phagocytes. Pneumococci also occur in over 90 different **types**: **I**, **II**, **III** and so on. The types differ in the chemistry of their polysaccharide capsule. Unlike the occasional shift of S -> R, the type of the organism is constant. Mice injected with a few S cells of, say, Type II pneumococci, will soon have their bodies teeming with descendant cells of the same type.

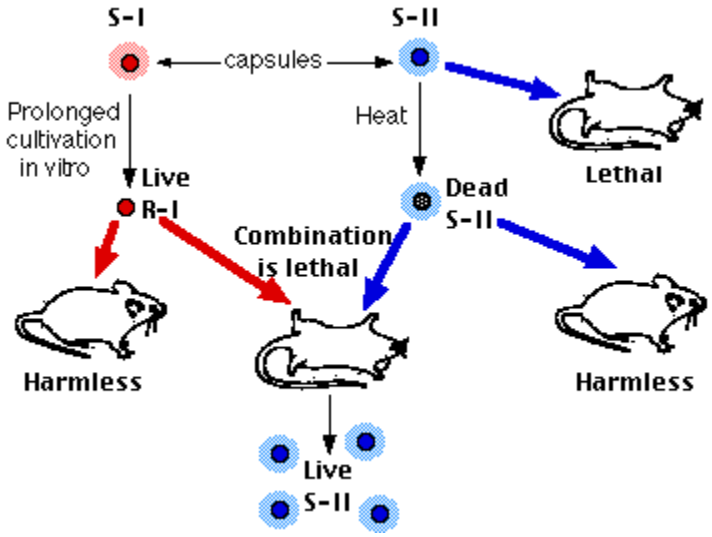


Figure 5.1.3: Griffith's experiment - injecting mice with Type II pneumococci

However, Griffith found that when **living** R cells (which should have been harmless) and **dead** S cells (which also should have been harmless) were injected together, the mouse became ill and **living S** cells could be recovered from its body. Furthermore, the **type** of the cells recovered from the mouse's body was determined by the type of the dead S cells. In the experiment shown above, injection of living R-I cells and dead S-II cells produced a dying mouse with its body filled with living S-II pneumococci. The S-II cells remained true to their new type. Something in the dead S-II cells had made a permanent change in the phenotype of the R-I cells. The process was named **transformation**.

Oswald Avery and his colleagues at The Rockefeller Institute in New York City eventually showed that the "something" was **DNA**. In pursuing Griffith's discovery, they found that they could bring about the same kind of transformation in vitro using an **extract** of the bacterial cells. They treated this extract with:

- an enzyme to destroy the polysaccharide of the capsule (S-III in their experiments)
- solvents to remove all lipids
- trypsin and chymotrypsin to destroy any residual proteins
- RNase to destroy RNA

They discovered that it did **not** destroy the ability of their extracts to transform the bacteria. However, treating the extracts with **DNase** to destroy the DNA in them did abolish their transforming activity. So DNA was the only material in the dead cells capable of transforming cells from one type to another. **DNA was the substance of genes.**

Although the chemical composition of the capsule is determined by genes, the relationship is indirect. DNA is transcribed into RNA and RNA is translated into proteins. The phenotype of the pneumococci — the chemical composition of the polysaccharide capsule — is determined by the particular enzymes (proteins) used in polysaccharide synthesis. Avery and his colleagues Colin MacLeod and Maclyn McCarty published their epoch-making findings on February 1, 1944. Unfortunately, the importance of their discovery was not sufficiently appreciated by scientists in general and the Nobel Committee in particular, and Avery died before their work could be honored with a Nobel Prize. (Nobel prizes are never given posthumously.)

Conjugation

Some bacteria, *E. coli* is an example, can transfer a portion of their chromosome to a recipient with which they are in direct contact. As the donor replicates its chromosome, the copy is injected into the recipient. At any time that the donor and recipient become separated, the transfer of genes stops. Those genes that successfully made the trip replace their equivalents in the recipient's chromosome.

Conjugation can only occur between cells of opposite mating types: the donor (or "male") carries a fertility factor ($\mathbf{F^+}$) and the recipient ("female") does not ($\mathbf{F^-}$). The fertility factor is a set of genes originally acquired from a plasmid and now integrated into the bacterial chromosome. It establishes the origin of replication for the chromosome. A portion of F is the "locomotive" that pulls the chromosome into the recipient cell and the rest of it is the "caboose".

In *E. coli*, about one gene gets across each second that the cells remain together. (So, it takes about 100 min for the entire genome (4377 genes) to make it. However, the process is easily interrupted so it is more likely that host genes close behind the leading F genes ("locomotive") will make it than those farther back. The "caboose" seldom makes it so failing to receive a complete F factor, the recipient cell continues to be "female".

The DNA that makes it across finds the homologous region on the female chromosome and replaces it (by a double crossover). By deliberately separating the cells (in a kitchen blender) at different times, the order and relative spacing of the genes can be determined. In this way, a genetic map — equivalent to the genetic maps of eukaryotes — can be made. However, here the map intervals are seconds, not centimorgans (cM).

Figure 5.1.4 shows the mechanism of conjugation in *E. coli* cells where the "male" lacks functional genes needed to synthesize the vitamin biotin and the amino acid methionine (Bio^-, Met^-) so these must be added to its culture medium. The "female" has those genes (Bio^+, Met^+) but has nonfunctional (mutant) genes that prevent it from being able to synthesize the amino acids threonine and leucine (Thr^-, Leu^-), so these must be added to its culture medium.

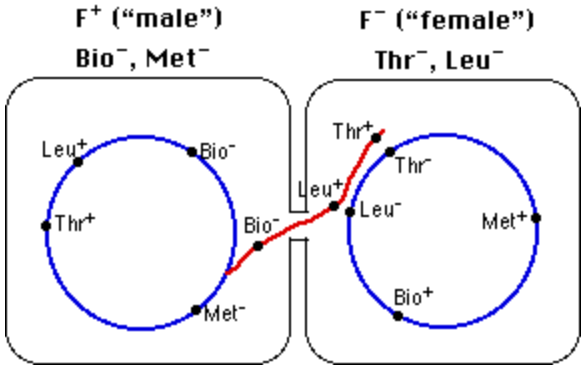


Figure 5.1.4: Conjugation in E. Coli cells

When cultured together, some female cells receive the functional **Thr** and **Leu** genes from the male donor. A double crossover enables them to replace the nonfunctional alleles. Now the cells now can grow on a "minimal" medium containing only glucose and salts.

Transduction

Bacteriophages are viruses that infect bacteria. In the process of assembling new virus particles, some host DNA may be incorporated in them. The virion head can hold only so much DNA so these viruses while still able to infect new host cells and may be unable to lyze them. Instead the hitchhiker bacterial gene (or genes) may be inserted into the DNA of the new host, replacing those already there and giving the host an altered phenotype. This phenomenon is called **transduction**.

Reductionist vs. holistic Approach

The understanding of complex systems almost always has to await unraveling the details of some simpler system. You may feel that trying to find out how one type of pneumococcus could be converted into another was an exceedingly specialized and esoteric pursuit. But Avery and his coworkers realized the broader significance of what they were observing and, in due course, the rest of the scientific world did as well. By electing to work with a well-defined system: the conversion of R forms of one type into S forms of a different type, these researchers made a discovery that has revolutionized biology and medicine. Attempting to understand the workings of complex systems by first understanding the workings of their parts is called **reductionism**. Some scientists (and many nonscientists) question the value of reductionism. They favor a **holistic** approach emphasizing the workings of the complete system.

However, the record speaks for itself. From skyscrapers to moon walks, to computer chips to the advances of modern medicine, progress comes from first understanding the properties of the parts that make up the whole. The late George Wald, who won the 1967 Nobel Prize in Physiology for his discoveries of the molecular basis of detecting light, once worried that his work was overly specialized — studying not vision, not the eye, not the whole retina, not even their rods and cones, but just the chemical reactions of their rhodopsins. But he came to realize "it is as though this were a very narrow window through which at a distance one can see only a crack of light. As one comes closer, the view grows wider and wider, until finally through this same window one is looking at the universe. I think this is the way it always goes in science, because science is all one. It hardly matters where one enters, provided one can come closer....".

Significance of Genetic Recombination in Bacteria

Transformation, conjugation, and transduction were discovered in the laboratory. How important are these mechanisms of genetic recombination in nature? The completion of the sequence of the entire genome of a variety of different bacteria (and archaea) suggest that genes have in the past moved from one species to another. This phenomenon is called **lateral gene transfer** (LGT). The remarkable spread of resistance to multiple antibiotics may have been aided by the transfer of resistance genes within populations and even between species. Many bacteria have enzymes that enable them to destroy foreign DNA that gets into their cells. It seem unlikely that these would be needed if that did not occur in nature. In any case, these **restriction enzymes** have provided the tools upon which the advances of molecular biology and the biotechnology industry depend.

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5.2: The Hershey - Chase Experiments

In 1952 (seven years after Avery's demonstration that genes were DNA), two geneticists: A. D. Hershey and Martha Chase, provided further proof. They worked with a DNA virus, called **T2**, which infects *E. coli* (and so is a bacteriophage). Figure 5.2.1 shows the essential elements of the infective cycle of DNA bacteriophages like T2. The virions attach to the surface of their host cell **(a)**. The proteins of the capsid inject the **DNA core** into the cell **(b)**. Once within the cell, some of the bacteriophage genes (the "early" genes) are transcribed (by the host's RNA polymerase) and translated (by the host's ribosomes, tRNA, etc.) to produce enzymes that will make many copies of the phage DNA and will turn off (even destroy) the host's DNA.

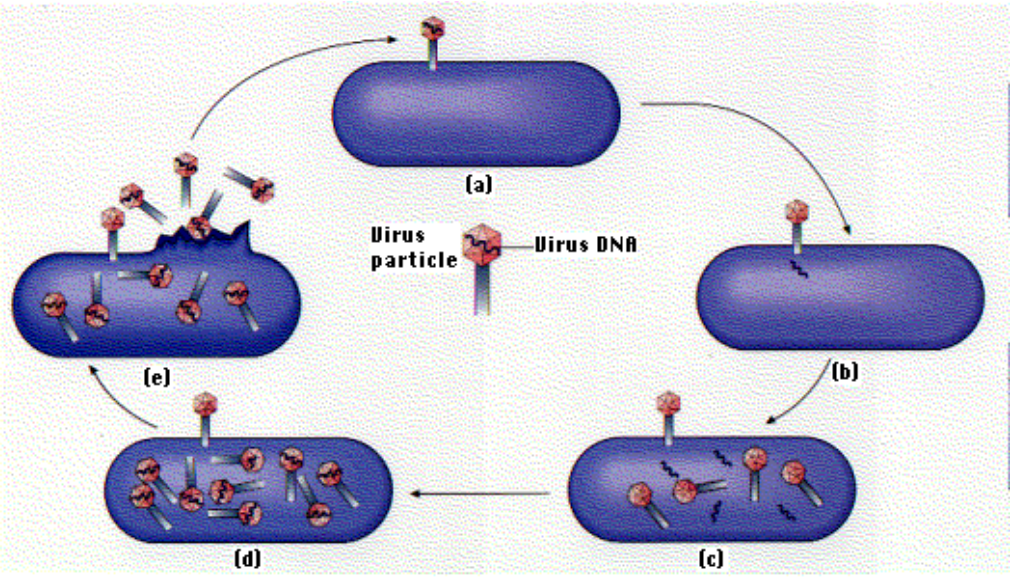


Figure 5.2.1: The Hershey - Chase Experiment

As fresh copies of phage DNA accumulate, other genes (the "late" genes) are transcribed and translated to form the proteins of the capsid **(c)**. The stockpile of DNA cores and capsid proteins are assembled into complete virions **(d)**. Another "late" gene is transcribed and translated into molecules of lysozyme. The lysozyme attacks the peptidoglycan wall (from the inside, of course). Eventually the cell ruptures and releases its content of virions ready to spread the infection to new host cells **(e)**.

Bacteriophages produced within bacteria growing in radioactive culture medium will themselves be radioactive. If radioactive sulfur atoms (^{35}S) are present, they will be incorporated into the protein coats of the bacteriophages since two of the amino acids — cysteine and methionine — contain sulfur (Figure 5.2.2). However, the DNA will be nonradioactive because there are no sulfur atoms in DNA. If radioactive phosphorus (^{32}P) is used instead, the DNA become radioactive — because of its many phosphorus atoms — but not the proteins.

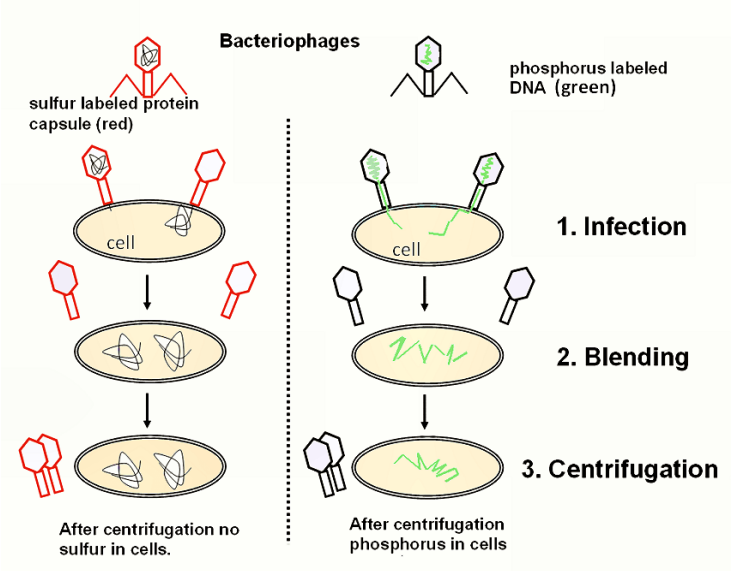


Figure 5.2.2: Diagram of the Hershey Chase experiment. (GFDL; Thomasione)

Hershey and Chase found that when bacteriophages containing ^{32}P (radioactive), were allowed to infect nonradioactive bacteria, all the infected cells became radioactive and, in fact, much of the radioactivity was passed on to the next generation of bacteriophages. However, when the bacteria were infected with bacteriophages labeled with ^{35}S, and then the virus coats removed (by whirling them in an electric blender), practically no radioactivity could be detected in the infected cells. From these experiments, it was clear that the DNA component of the bacteriophages is injected into the bacterial cell while the protein component remains outside. However, it is the injected component — DNA — that is able to direct the formation of new virus particles **complete** with protein coats. So here is further proof that **genes are DNA**.

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5.3: The Double Helix of DNA

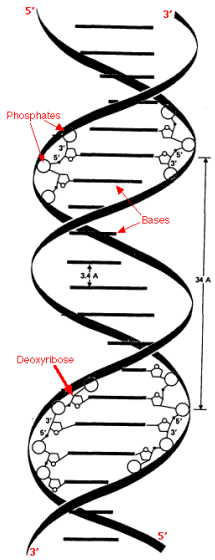


Figure 5.3.1: Double Helix of DNA

This structure of DNA was worked out by **Francis Crick** and **James D. Watson** in 1953. It revealed how DNA - the molecule that Avery had shown was the physical substance of the genes. It could be replicated and so passed on from generation to generation. For this epochal work, they shared a Nobel Prize in 1962.

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5.4: Base Pairing in DNA and RNA

Rules of Base Pairing

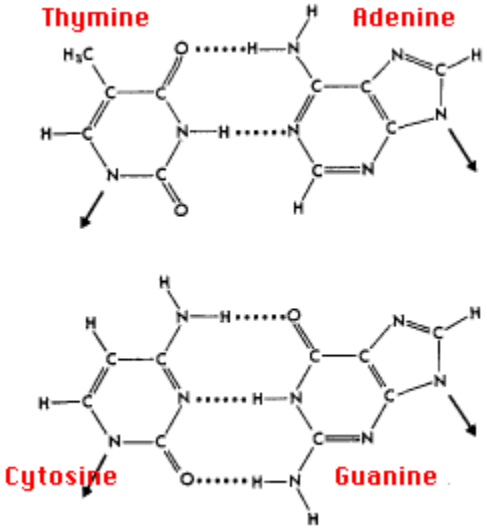


Figure 5.4.1: Base Pairing

The rules of base pairing (or nucleotide pairing) are:

- A with **T**: the purine **adenine** (A) always pairs with the pyrimidine **thymine** (T)
- **C** with **G**: the pyrimidine **cytosine** (C) always pairs with the purine **guanine** (G)

This is consistent with there not being enough space (20 Å) for two purines to fit within the helix and too much space for two pyrimidines to get close enough to each other to form hydrogen bonds between them. But why not A with C and G with T? The answer: only with A & T and with C & G are there opportunities to establish **hydrogen bonds** (shown here as dotted lines) between them (two between A & T; three between C & G). These relationships are often called the rules of **Watson-Crick** base pairing, named after the two scientists who discovered their structural basis.

Table 5.4.1: Relative Proportions (%) of Bases in DNA

Organism	A	T	G	C
Human	30.9	29.4	19.9	19.8
Chicken	28.8	29.2	20.5	21.5
Grasshopper	29.3	29.3	20.5	20.7
Sea Urchin	32.8	32.1	17.7	17.3
Wheat	27.3	27.1	22.7	22.8
Yeast	31.3	32.9	18.7	17.1
E. coli	24.7	23.6	26.0	25.7

The rules of base pairing tell us that if we can "read" the sequence of nucleotides on one strand of DNA, we can immediately deduce the complementary sequence on the other strand. The rules of base pairing explain the phenomenon that whatever the amount of adenine (A) in the DNA of an organism, the amount of thymine (T) is the same (called **Chargaff's rule**). Similarly, whatever the amount of guanine (G), the amount of cytosine (C) is the same. The C+G:A+T ratio varies from organism to organism, particularly among the bacteria, but within the limits of the experimental error, A=T and C=G.

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5.5: DNA Replication

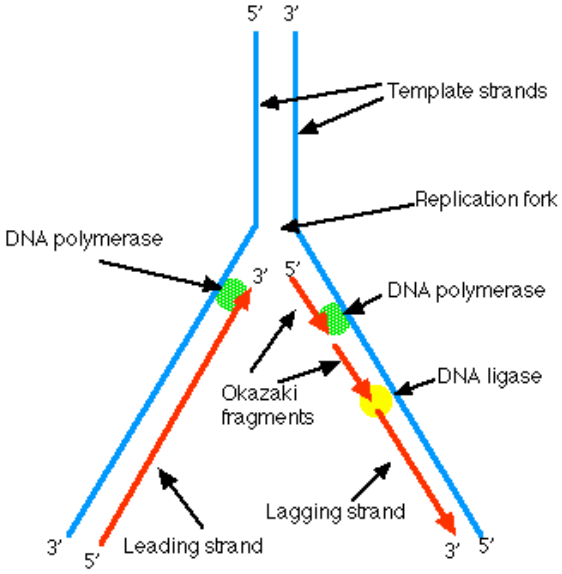


Figure 5.5.3: Control of Replication

With their multiple origins, how does the **eukaryotic cell** know which origins have been already replicated and which still await replication?

An observation: When a cell in G_2 of the cell cycle is fused with a cell in S phase, the DNA of the G_2 nucleus does not begin replicating again even though replication is proceeding normally in the S-phase nucleus. Not until mitosis is completed, can freshly-synthesized DNA be replicated again.

Two control mechanisms have been identified — one **positive** and one **negative**. This redundancy probably reflects the crucial importance of precise replication to the integrity of the genome.

Licensing: positive control of replication

In order to be replicated, each origin of replication must be bound by:

- an **O**rigin **R**ecognition **C**omplex of proteins (**ORC**). These remain on the DNA throughout the process.
- Accessory proteins called **licensing factors**. These accumulate in the nucleus during G_1 of the cell cycle. They include:
  - Cdc-6 and Cdt-1, which bind to the **ORC** and are essential for coating the DNA with
  - MCM proteins. Only DNA coated with MCM proteins (there are 6 of them) can be replicated.

Once replication begins in S phase,

- Cdc-6 and Cdt-1 leave the ORCs (the latter by ubiquination and destruction in proteasomes).
- The MCM proteins leave in front of the advancing replication fork.

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5.6: The Meselson - Stahl Experiment

DNA Replication is Semiconservative

The structure of DNA suggested to Watson and Crick the mechanism by which DNA — hence genes — could be copied faithfully. They proposed that when the time came for DNA to be replicated, the two strands of the molecule

- separated from each other but
- remained intact as each served as the template for the synthesis of
- a complementary strand.

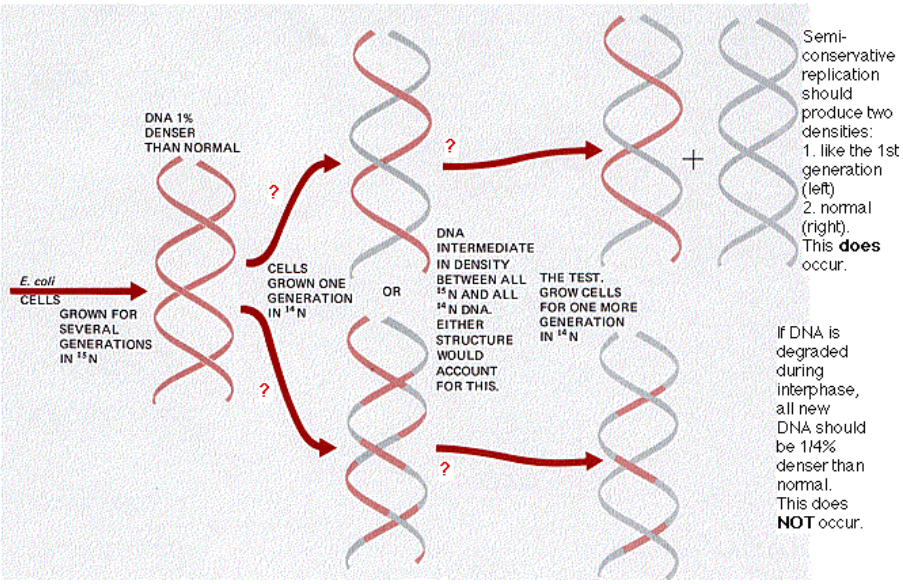


Figure 5.6.1 Meselson - Stahl experiment interpretation

As this interpretative figure indicates, their results show that DNA molecules are not degraded and reformed from free nucleotides between cell divisions, but instead, each original strand remains intact as it builds a complementary strand from the nucleotides available to it. This is called semiconservative replication because each daughter DNA molecule is one-half "old" and one-half "new".

> **Immortal strands.** Note that the "old" strand (the red one in the top half of the figure) is immortal because — barring mutations or genetic recombination — it will continue to serve as an unchanging template down through the generations.

E. coli is a bacterium, but semiconservative replication of DNA also occurs in eukaryotes. And because each DNA molecule in a eukaryote is incorporated in **one chromosome**, the replication of entire chromosomes is semiconservative as well. This also means that the eukaryotic chromosome contains one "immortal strand" of DNA.

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5.7: Restriction Enzymes

Restriction enzymes are DNA-cutting enzymes found in bacteria (and harvested from them for use). Because they cut within the molecule, they are often called restriction endonucleases. To be able to sequence DNA, it is first necessary to cut it into smaller fragments. Many DNA-digesting enzymes (like those in your pancreatic fluid) can do this, but most of them are no use for sequence work because they cut each molecule randomly. This produces a heterogeneous collection of fragments of varying sizes. What is needed is a way to cleave the DNA molecule at a few precisely-located sites so that a small set of homogeneous fragments are produced. The tools for this are the restriction endonucleases. The rarer the site it recognizes, the smaller the number of pieces produced by a given restriction endonuclease.

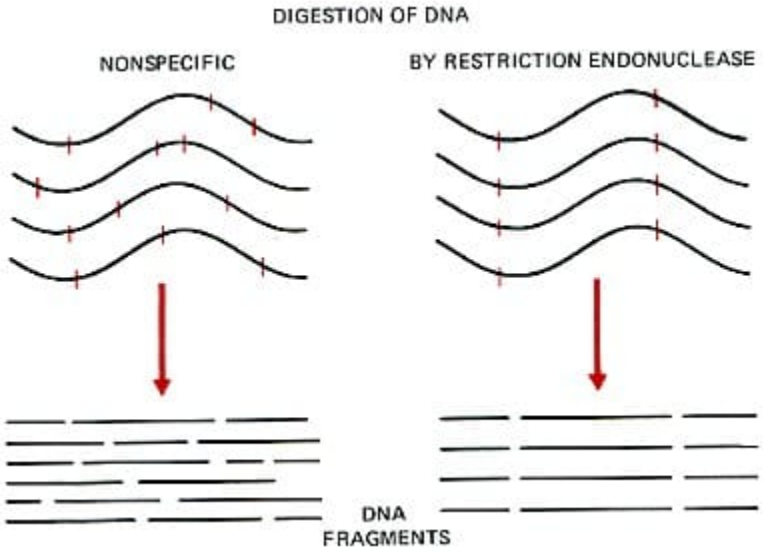


Figure 5.7.1: Restriction Digest

A restriction enzyme recognizes and cuts DNA only at a particular sequence of nucleotides. For example, the bacterium *Hemophilus aegypticus* produces an enzyme named HaeIII that cuts DNA wherever it encounters the sequence

5'GGCC3'

3'CCGG5'

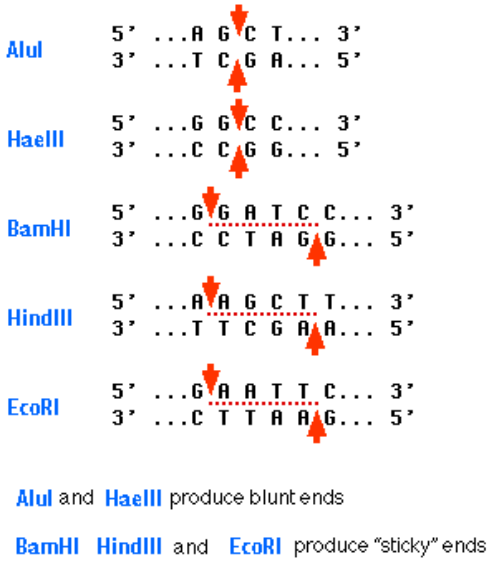


Figure 5.7.2: Restriction Enzymes

The cut is made between the adjacent G and C. This particular sequence occurs at 11 places in the circular DNA molecule of the virus φX174. Thus treatment of this DNA with the enzyme produces 11 fragments, each with a precise length and nucleotide sequence. These fragments can be separated from one another and the sequence of each determined. HaeIII and AluI cut straight across the double helix producing "blunt" ends. However, many restriction enzymes cut in an offset fashion. The ends of the cut have an overhanging piece of single-stranded DNA. These are called "sticky ends" because they are able to form base pairs with any DNA molecule that contains the complementary sticky end. Any other source of DNA treated with the same enzyme will produce such molecules. Mixed together, these molecules can join with each other by the base pairing between their sticky ends. The union can be made permanent by another enzyme, a DNA ligase, that forms covalent bonds along the backbone of each strand. The result is a molecule of recombinant DNA (rDNA).

The ability to produce recombinant DNA molecules has not only revolutionized the study of genetics, but has laid the foundation for much of the biotechnology industry. The availability of human insulin (for diabetics), human factor VIII (for males with hemophilia A), and other proteins used in human therapy all were made possible by recombinant DNA.

Artificial Restriction Enzymes

In addition to the many natural restriction enzymes isolated from bacteria and archaea, it is now possible to synthesize artificial restriction enzymes that cut DNA at any desired sequence. Examples:

- zinc-finger nucleases
- TALENs
- CRISPR RNA molecules

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5.8: DNA Sequencing by the Dideoxy Method

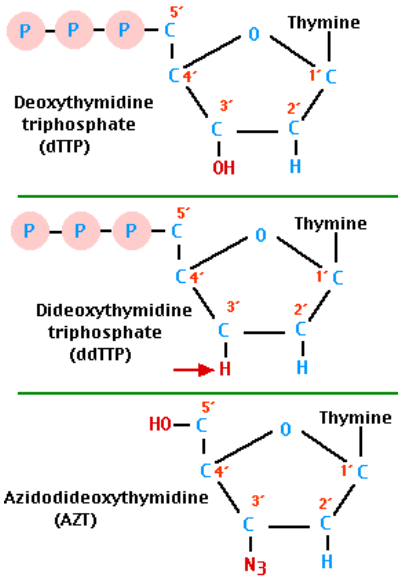


Figure 5.8.2: Sample DNA Sequencing

The DNA to be sequenced is prepared as a single strand. This template DNA is supplied with

- a mixture of all four **normal** (deoxy) nucleotides in ample quantities
  - dATP
  - dGTP
  - dCTP
  - dTTP
- a mixture of all four **dideoxy**nucleotides, each present in limiting quantities and each labeled with a "tag" that fluoresces a different color:
  - **ddATP**
  - **ddGTP**
  - **ddCTP**
  - **ddTTP**
- DNA polymerase I

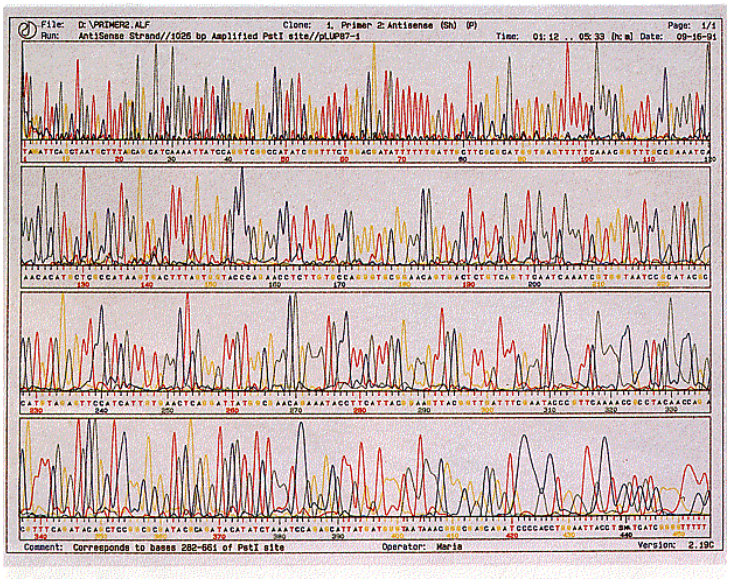


Figure 5.8.3 example of a DNA sequence (455 nucleotides of the lysU gene of E. coli) courtesy of Pharmacia Biotech Inc., Piscataway, NJ

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5.9: Genome Sizes

The genome of an organism is the complete set of genes specifying how its phenotype will develop (under a certain set of environmental conditions). In this sense, then, **diploid** organisms (like ourselves) contain two genomes, one inherited from our mother, the other from our father. The table below presents a selection of representative genome sizes from the rapidly-growing list of organisms whose genomes have been sequenced.

Table of Genome Sizes (haploid)

	Base pairs	Genes	Notes
φX174	5,386	11	virus of E. coli
Human mitochondrion	16,569	37	
Nasuia deltocephalinicola	112,091	137	smallest genome yet found in a bacterium. This β-proteobacterium lives in a mutualistic relationship within a special organ of an insect (a leaf hopper) which it supplies with essential amino acids.
Epstein-Barr virus (EBV)	172,282	80	causes mononucleosis
nucleomorph of *Guillardia theta*	551,264	511	all that remains of the nuclear genome of a red alga (a eukaryote) engulfed long ago by another eukaryote
Mycoplasma genitalium	580,073	525	two of the smallest true organisms
Mycoplasma pneumoniae	816,394	679	
Rickettsia prowazekii	1,111,523	834	bacterium that causes epidemic typhus
Treponema pallidum	1,138,011	1,039	bacterium that causes syphilis
Pelagibacter ubique	1,308,759	1,354	smallest genome yet found in a **free-living** organism (marine α-proteobacterium)
Helicobacter pylori	1,667,867	1,589	chief cause of stomach ulcers (not stress and diet)
Methanocaldococcus jannaschii	1,664,970	1,783	These unicellular microbes look like typical bacteria but their genes are so different from those of either bacteria or eukaryotes that they are classified in a third kingdom: **Archaea**.
Aeropyrum pernix	1,669,695	1,885	
Methanothermobacter thermoautotrophicus	1,751,377	2,008	
Streptococcus pneumoniae	2,160,837	2,236	the pneumococcus
Pandoravirus	2,473,870	2556	A **virus** (of an amoeba) with a genome larger than that of the bacteria and archaea above and about the same as that of some parasitic **eukaryotes.**
Listeria monocytogenes	2,944,528	2,926	2,853 of these encode proteins; the rest RNAs
Synechocystis	3,573,470	4,003	a marine cyanobacterium ("blue-green alga")
E. coli K-12	4,639,221	4,377	4,290 of these genes encode proteins; the rest RNAs
E. coli O157:H7	5.44×10^6	5,416	strain that is pathogenic for humans; has 1,346 genes not found in E. coli K-12

	Base pairs	Genes	Notes
Schizosaccharomyces pombe	12,462,637	4,929	Fission yeast. A **eukaryote** with fewer genes than the three bacteria below.
Agrobacterium tumefaciens	4,674,062	5,419	Useful vector for making transgenic plants; shares many genes with *Sinorhizobium meliloti*
Pseudomonas aeruginosa	6.3×10^6	5,570	Increasingly common cause of opportunistic infections in humans.
Sinorhizobium meliloti	6,691,694	6,204	The rhizobial symbiont of alfalfa. Genome consists of one chromosome and 2 large plasmids.
Saccharomyces cerevisiae	12,495,682	5,770	Budding yeast. A eukaryote.
Neurospora crassa	38,639,769	10,082	Plus 498 RNA genes.
Thalassiosira pseudonana	34.5×10^6	11,242	A diatom. Plus 144 chloroplast and 40 mitochondrial genes encoding proteins
Naegleria gruberi	41×10^6	15,727	This free-living unicellular organism lives as both an amoeboid and a flagellated form. 4,133 of its genes are also found in other eukaryotes suggesting that they were present in the common ancestor of all eukaryotes. The great variety of functions encoded by these genes also suggests that the common ancestor of all eukaryotes was itself as complex as many of the present-day unicellular members.
Drosophila melanogaster	122,653,977	~17,000	the "fruit fly"
Caenorhabditis elegans	100,258,171	21,733	
Humans	3.3×10^9	~21,000	
Tetraodon nigroviridis (a pufferfish)	3.42×10^8	27,918	Although Tetraodon seems to have more protein-encoding genes than we do, it has much less non-coding DNA so its total genome is about a tenth the size of ours.
Mouse	2.8×10^9	~23,000	
Amphibians	10^9–10^{11}	?	
Arabidopsis thaliana	0.135×10^9	27,407	a flowering plant (angiosperm) with one of the smallest genomes known in the plant kingdom.
Picea abies	19.6×10^9	28,354	the Norway spruce, a conifer (gymnosperm). Even though it has only ~900 more genes than Arabidopsis, it has 145 times as much DNA. Most of this appears to be derived from transposons.
Psilotum nudum	2.5×10^{11}	?	

Even though *Psilotum nudum* (sometimes called the "whisk fern") is a far simpler plant than Arabidopsis (it has no true leaves, flowers, or fruit), it has 3000 times as much DNA. No one knows why, but 80% or more of it is repetitive DNA containing no genetic information. This is also the case for some amphibians, which contain 30 times as much DNA as we do, but certainly are not 30 times as complex. The total amount of DNA in the haploid genome is called its C value. The lack of a consistent relationship between the C value and the complexity of an organism (e.g., amphibians vs. mammals) is called the C value paradox.

Not all genes are Indispensable

The scientists at The Institute for Genomic Research (now known as the J. Craig Venter Institute) who determined the

Mycoplasma genitalium sequence have followed this work by systematically destroying its genes (by mutating them with insertions) to see which ones are essential to life and which are dispensable. Of the 485 protein-encoding genes, they have concluded that only 381 of them are essential to life. In other words, the loss of any one of the 381 is lethal; the loss of any one of the others is not. (This is not to say that all the organism needs are those 381 — see "A Minimal Genome?" below.)

Using similar techniques, three groups have recently found that only about 10% of the genes in the human genome (~2000 of them) must be present for human cells to grow successfully in culture. These genes encode proteins for such essential functions as controlling the cell cycle, DNA replication, DNA transcription and RNA translation. The cells can tolerate the loss of any one of the other ~18,000 genes. Thus the human genome appears to have redundant pathways that can often compensate for the loss of a single gene at least for cells growing in culture. Probably others will turn out to be essential for the development and functioning of the various types of differentiated cells in the intact body.

A Minimal Genome?

In March of 2016, workers at the J. Craig Venter Institute reported that they had created a strain of *mycoplasma* containing only 473 genes. This synthetic organism, which grows vigorously in culture, now holds the record for the smallest genome of a free-living organism.

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5.10: The Human Genome Projects

Shortly after their press conferences, the two groups that had been striving for several years to map the human genome published their findings:

- The **International Human Genome Sequencing Consortium** (IHGSC) in the 15 February 2001 issue of **Nature**
- **Celera Genomics**, a company in Rockville, Maryland, in the 16 February issue of **Science**

These achievements were monumental, but before we examine them, let us be clear as to what they were not.

What was not found

- Neither group had determined the complete sequence of the human genome. Each of our chromosomes is a single molecule of DNA. Some day the sequence of base pairs in each will be known from one end to the other. But in 2001, thousands of gaps remained to be filled. What they had done was present a series of draft sequences that represented about 90% (probably the most interesting 90%) of the genome.
- Even taken together, the results did not provide an accurate count of the number of protein-encoding genes in our genome (in contrast to such genomes as those of mitochondrial DNA, the Epstein-Barr virus and many of the bacterial genomes.
  One reason: the large number and large size of the introns that split these genes make it difficult to recognize the open reading frames (ORFs) that encode proteins.

The number of genes were much smaller than predicted

The two groups came up with slightly different estimates of the number of protein-encoding genes, but both in the range of 30 to 38 thousand:

- barely two times larger than the genomes of
  - Drosophila (~17,000 genes)
  - *C. elegans* (<22,000 genes)
- and representing only 1– 2% of the total DNA in the cell;
- and a third of the 100,000 genes that many had predicted would be found.
- (By 2011, the number had been reduced to some 21,000.)

Are the tiny roundworm and fruit fly almost as complex as we are?

Probably not, although we share many homologous genes (called "**orthologs**") with both these animals. But many of our protein-encoding genes produce more than one protein product (e.g., by alternative splicing of the primary transcript of the gene). On average, each of our ORFs produces 2 to 3 different proteins. So the human "**proteome**" (our total number of proteins) may be 10 or more times larger than that of the fruit fly and roundworm.

A larger proportion of our genome encodes transcription factors and is dedicated to **control elements** (e.g., enhancers) to which these transcription factors bind. The combinatorial use of these elements probably provides much greater flexibility of gene expression than is found in Drosophila and *C. elegans*.

Gene diversity and density

There are some giants such as *dystrophin* with its 79 exons spread over 2.4 million base pairs of DNA and titin whose 363 exons can encode a single protein with as many as ~38,000 amino acids. The average human gene contains 4 exons totaling 1,350 base pairs and thus encodes an average protein of 450 amino acids. The density of genes on the different chromosomes varies from 23 genes per million base pairs on chromosome 19 (for a total of 1,400 genes) to only 5 genes per million base pairs on chromosome 13.

Humans have many genes not found in invertebrates

Humans, and presumably most vertebrates, have genes not found in invertebrate animals like Drosophila and *C. elegans*. These include genes encoding:

- **antibodies** and **T cell receptors for antigen** (TCRs)
- the transplantation antigens of the **major histocompatibility complex (MHC)** (HLA, the MHC of humans)
- cell-signaling molecules including the many types of cytokines
- the molecules that participate in blood clotting
- mediators of apoptosis. Although these proteins occur in Drosophila and *C. elegans*, we have a much richer assortment of them.

Gene Duplication

Both groups added to the list of human genes that have arisen by repeated duplication (e.g., by **unequal crossing over**) from a single precursor gene; for examples, the genes (several hundred) for olfactory receptors and the various globin genes.

Repetitive DNA

Both groups verified the presence of large amounts of repetitive DNA. In fact, this DNA — with similar sequences occurring over and over — is one of the main obstacles to assembling the DNA sequences in proper order.

- LINES (long interspersed elements)
- SINES (short interspersed elements) including Alu elements
- Retrotransposons
- DNA transposons

All told, repetitive DNA probably accounts for over 50% of our total genome.

What remains to be done?

- Keep looking for genes.

  As of March 2010, 19,956 protein-encoding genes had been positively identified, but there probably are a thousand or more still to be found.

- Determine the human **proteome**; that is, the total complement of proteins we synthesize.
- Analyze how clusters of genes are coordinately expressed
  - in various types of cells
  - at different times in the life of a cell.

  Such analysis will benefit greatly from the availability to gene chip technology and will also help us to understand how such a modest increase in gene number from Drosophila to humans could produce such a different outcome!
- Determine the genomes of other vertebrates.

  This will not only help us recognize more human genes but will give us insight into what makes us unique.

  Already we know that large sections of our genome have closely-related homologs in the **mouse**.

  Examples:
  - The collection of genes — and even their order — on human chromosome 17 matches closely those of mouse chromosome 11. The same is true of human chromosome 20 and mouse chromosome 2.
  - Humans and mice (also rats) share several hundred **absolutely identical** stretches of DNA extending for 200–800 base pairs.
    - Some are present in the exons of genes, especially genes involved in RNA processing.
    - Some are found in or near the introns of genes, especially genes encoding proteins involved in DNA transcription.
    - Some are found between genes — especially those, like *Pax6*, essential to embryonic development — and may serve as enhancers.

To have avoided any mutations for 60 million years since humans and rodents went their separate evolutionary ways suggest that these regions perform functions absolutely essential to mammalian life.

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5.11: The Human and Chimpanzee Genomes

Now that the genomes of both the human and the chimpanzee have been determined, it is possible to make more direct comparisons between the two species.

Comparison results

Their genomes are 98.8% identical (between any two humans — picked at random — the figure is closer to 99.5%).

- Another gene product that differs between the two species is the protein **FoxP2**. FoxP2 is a transcription factor. Rare humans with only one copy of the gene (*FOXP2*) have severe language defects.

  The human *FOXP2* gene differs from that of the chimp by 5 nucleotides, 2 of which result in non-synonymous codons encoding 2 different amino acids in the protein. The human protein differs from that in the mouse by only 3 amino acids. When you consider that we shared a common ancestor with mice over 60 million years ago but with the champanzee only about 6 million years ago, it is tempting to think that these recent changes in the human gene are related to the acquisition of language.

- While there are only small (~1%) coding differences in their genes, their genomes differ in other ways.
  - Many insertions and deletions ("indels") and
  - many gene duplications

  are found in one species but not the other. Later work has revealed that of 510 chimpanzee sequences that are deleted in the human genome, only one occurs in the coding region of a gene. The others are found in introns or between genes, and at least some of these occur in gene-regulatory regions like enhancers.
- Many single-nucleotide differences create different splicing sites so alternative splicing can produce substantial differences in the proteins of two species.

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5.12: Pyrosequencing

In laboratories around the world there is an intense desire to sequence more genomes.

- those of a wide variety of organisms to aid in establishing evolutionary relationships;
- those of pooled populations of microorganisms in, for examples, sea water, soil, the large intestine;
- other humans to look for genes that predispose to disease and genetic patterns in various ethnic groups.

All of the sequenced genomes listed in Genome Sizes were determined using the dideoxy method invented by Frederick Sanger and described elsehwere. However, now a great effort is being expended to find ways to sequence DNA more rapidly (and more cheaply).

The Genome Sequencer

Several new methods are being developed and one is already commercially available (the Genome Sequencer 20 System). Its method is called pyrosequencing or sequencing by synthesis. It works like this.

- The DNA to be sequenced is broken up into fragments of ~100 base pairs and denatured to form single-stranded DNA (ssDNA).
- Single ssDNA fragments are attached to microscopic beads, which are separated from each other.
- The polymerase chain reaction (PCR) is run on each bead so that each becomes coated with ~ 10 million identical copies of that fragment.
- The beads are placed singly into separate, microscopic wells (~200,000 of them).
- Each well receives a cocktail of reagents:
  - DNA polymerase — for adding deoxyribonucleotides to the ssDNA
  - adenosine phosphosulfate (APS)
  - ATP sulfurylase — an enzyme that forms ATP from adenosine phosphosulfate (APS) and pyrophosphate (PPi)
  - luciferin
  - luciferase - an ATPase that catalyzes the conversion of luciferin to oxyluciferin with the liberation of light

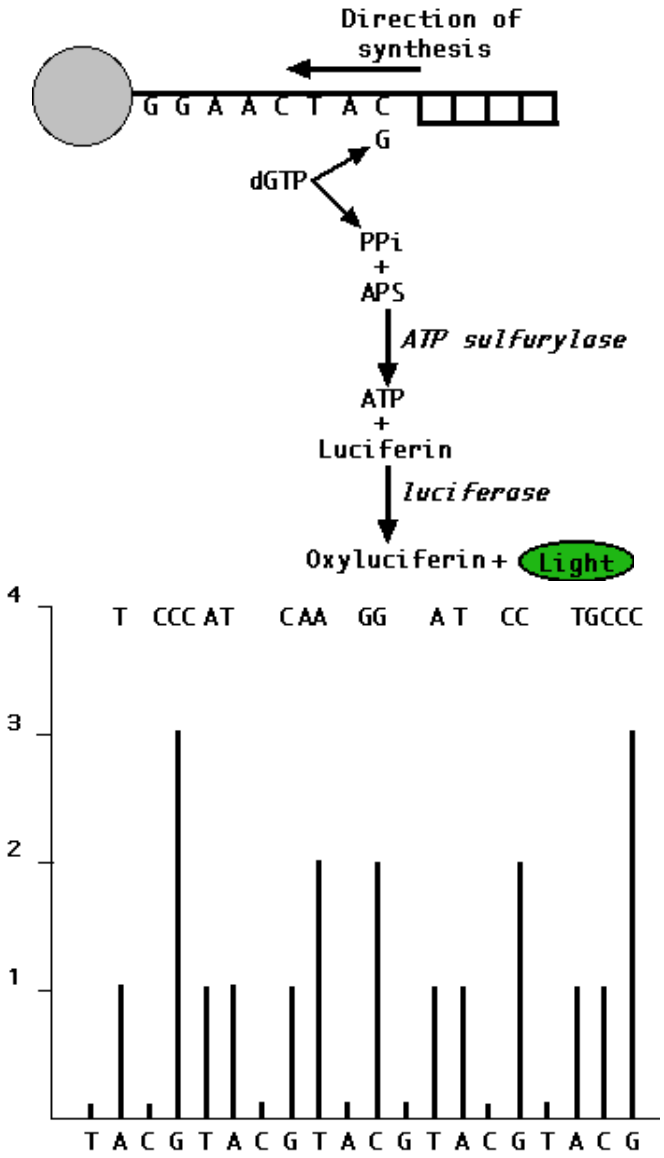


Figure 5.12.1 Pyrosequencing run and the data produced by a single well

The sequencing run:

- Each of the thousands of wells is flooded with one four deoxyribonucleotides, dTTP, dCTP, and dGTP, but instead of dATP (which would trigger the luciferin reaction), deoxyadenosine alpha-thiotriphosphate (dATPαS) is used instead. DNA polymerase ignores the difference and uses it whenever a T is encountered on the ssDNA template, but luciferase doesn't recognize to it.
- In any well where the complementary nucleotide is present at the 3' end of the template, the nucleotide is added and pyrophosphate is liberated.
- The amount of light is proportional to the number of that nucleotide added. So if, for example, the incoming nucleotide is dGTP, and there is a string of 3 Cs on the template, the light emitted will be 3 times brighter than if only one C is present.
- A detector picks up the light (if any) from each well and the data are recorded.
- Then each of the remaining 3 nucleotides are added in sequence.
- Then the sequence of 4 additions is repeated until synthesis is complete.

The above diagram also shows the type of data produced in a single well. The height of the peak of light production gives the number of additions that occurred when a particular nucleotide was added (bottom). Computer software then displays the template sequence (top) for each of the thousands of different fragments sequenced. With this technology, as many as 20 million base pairs of genome sequence can be learned in an instrument run of less than 6 hours.

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5.13: DNA Repair

DNA in the living cell is subject to many chemical alterations (a fact often forgotten in the excitement of being able to do DNA sequencing on dried and/or frozen specimens). If the genetic information encoded in the DNA is to remain uncorrupted, any chemical changes must be corrected.

A failure to repair DNA produces a mutation.

The recent publication of the human genome has already revealed 130 genes whose products participate in DNA repair. More will probably be identified soon.

Agents that Damage DNA

- Certain wavelengths of **radiation including** ionizing radiation such as gamma rays and X-rays and **ultraviolet rays**, especially the UV-C rays (~260 nm) that are absorbed strongly by DNA but also the longer-wavelength UV-B that penetrates the ozone shield.
- Highly-reactive **oxygen radicals** produced during normal cellular respiration as well as by other biochemical pathways.
- Chemicals in the **environment**
  - many hydrocarbons, including some found in cigarette smoke
  - some plant and microbial products, e.g. the aflatoxins produced in moldy peanuts
- Chemicals used in **chemotherapy**, especially chemotherapy of cancers

Types of DNA Damage

1. All four of the bases in DNA (**A, T, C, G**) can be covalently modified at various positions.
   - One of the most frequent is the loss of an amino group ("deamination") - resulting, for example, in a **C** being converted to a **U**.
2. **Mismatches** of the normal bases because of a failure of proofreading during DNA replication.
   - Common example: incorporation of the pyrimidine **U** (normally found only in RNA) instead of **T**.
3. **Breaks** in the backbone.
   - Can be limited to one of the two strands (a single-stranded break, **SSB**) or on **both strands** (a **d**ouble-**s**tranded **b**reak (**DSB**). Ionizing radiation is a frequent cause, but some chemicals produce breaks as well.
4. **Crosslinks** Covalent linkages can be formed between bases
   - on the same DNA strand ("intrastrand") or
   - on the opposite strand ("interstrand").

   Several chemotherapeutic drugs used against cancers crosslink DNA

Repairing Damaged Bases

Damaged or inappropriate bases can be repaired by several mechanisms:

- **Direct chemical reversal** of the damage
- **Excision Repair**, in which the damaged base or bases are removed and then replaced with the correct ones in a localized burst of DNA synthesis. There are three modes of excision repair, each of which employs specialized sets of enzymes.
  1. **Base Excision Repair** (**BER**)
  2. **Nucleotide Excision Repair** (**NER**)
  3. **Mismatch Repair** (**MMR**)

The 2015 Nobel Prize in chemistry was shared by three researchers for their pioneering work in DNA repair: Tomas Lindahl (BER), Aziz Sancar (NER), and Paul Modrich (MMR).

Direct Reversal of Base Damage

Perhaps the most frequent cause of point mutations in humans is the spontaneous addition of a methyl group (CH_3-) (an example of alkylation) to Cs followed by deamination to a T. Fortunately, most of these changes are repaired by enzymes, called glycosylases, that remove the mismatched T restoring the correct C. This is done without the need to break the DNA backbone (in contrast to the mechanisms of excision repair described below).

Some of the drugs used in cancer chemotherapy ("chemo") also damage DNA by alkylation. Some of the methyl groups can be removed by a protein encoded by our ***MGMT*** gene. However, the protein can only do it once, so the removal of each methyl group requires another molecule of protein.

This illustrates a problem with direct reversal mechanisms of DNA repair: they are quite wasteful. Each of the myriad types of chemical alterations to bases requires its own mechanism to correct. What the cell needs are more general mechanisms capable of correcting all sorts of chemical damage with a limited toolbox. This requirement is met by the mechanisms of **excision repair**.

Base Excision Repair (BER)

The steps and some key players:

1. removal of the damaged base (estimated to occur some 20,000 times a day in each cell in our body!) by a DNA glycosylase. We have at least 8 genes encoding different DNA glycosylases each enzyme responsible for identifying and removing a specific kind of base damage.
2. removal of its deoxyribose phosphate in the backbone, producing a gap. We have two genes encoding enzymes with this function.
3. replacement with the correct nucleotide. This relies on **DNA polymerase beta**, one of at least 11 DNA polymerases encoded by our genes.
4. ligation of the break in the strand. Two enzymes are known that can do this; both require ATP to provide the needed energy.

Nucleotide Excision Repair (NER)

NER differs from BER in several ways.

- It uses different enzymes.
- Even though there may be only a single "bad" base to correct, its nucleotide is removed along with many other adjacent nucleotides; that is, NER removes a large "patch" around the damage.

The steps and some key players:

1. The damage is recognized by one or more protein factors that assemble at the location.
2. The DNA is unwound producing a "bubble". The enzyme system that does this is **Transcription Factor IIH**, **TFIIH**, (which also functions in normal transcription).
3. Cuts are made on both the 3' side and the 5' side of the damaged area so the tract containing the damage can be removed.
4. A fresh burst of DNA synthesis — using the intact (opposite) strand as a template — fills in the correct nucleotides. The DNA polymerases responsible are designated polymerase **delta** and **epsilon**.
5. A **DNA ligase** covalently inserts the fresh piece into the backbone.

Xeroderma Pigmentosum (XP)

XP is a rare **inherited** disease of humans which, among other things, predisposes the patient to

- pigmented lesions on areas of the skin exposed to the sun and
- an elevated incidence of skin cancer.

It turns out that XP can be caused by mutations in any one of several genes — all of which have roles to play in **NER**. Some of them:

- ***XPA***, which encodes a protein that binds the damaged site and helps assemble the other proteins needed for NER.
- ***XPB*** and ***XPD***, which are part of TFIIH. Some mutations in XPB and XPD also produce signs of premature aging. [Link]
- ***XPF***, which cuts the backbone on the 5' side of the damage
- ***XPG***, which cuts the backbone on the 3' side.

Transcription-Coupled NER

Nucleotide-excision repair proceeds most rapidly in cells whose genes are being actively transcribed on the DNA strand that is serving as the template for transcription. This enhancement of NER involves XPB, XPD, and several other gene products. The genes for two of them are designated ***CSA*** and ***CSB*** (mutations in them cause an inherited disorder called **Cockayne's syndrome**).

The CSB product associates in the nucleus with **RNA polymerase II**, the enzyme responsible for synthesizing **messenger RNA** (mRNA), providing a molecular link between transcription and repair. One plausible scenario: If RNA polymerase II, tracking along the template (antisense) strand), encounters a damaged base, it can recruit other proteins, e.g., the CSA and CSB proteins, to make a quick fix before it moves on to complete transcription of the gene.

Mismatch Repair (MMR)

Mismatch repair deals with correcting mismatches of the **normal bases**; that is, failures to maintain normal Watson-Crick base pairing (A•T, C•G). It can enlist the aid of enzymes involved in both base-excision repair (BER) and nucleotide-excision repair (NER) as well as using enzymes specialized for this function.

- **Recognition** of a mismatch requires several different proteins including one encoded by ***MSH2***.
- **Cutting** the mismatch out also requires several proteins, including one encoded by ***MLH1***.

Mutations in either of these genes predisposes the person to an inherited form of colon cancer. So these genes qualify as tumor suppressor genes.

How does the MMR system know which is the incorrect nucleotide?

In *E. coli*, certain adenines become methylated shortly after the new strand of DNA has been synthesized. The MMR system works more rapidly, and if it detects a mismatch, it assumes that the nucleotide on the already-methylated (parental) strand is the correct one and removes the nucleotide on the freshly-synthesized daughter strand. How such recognition occurs in mammals is not yet known.

Synthesis of the repair patch is done by **DNA polymerase delta**. Cells also use the MMR system to enhance the fidelity of recombination; i.e., assure that only homologous regions of two DNA molecules pair up to cross over and recombine segments (e.g., in meiosis).

Repairing Strand Breaks

Ionizing radiation and certain chemicals can produce both single-strand breaks (**SSB**s) and double-strand breaks (**DSB**s) in the DNA backbone.

Single-Strand Breaks (SSBs)

Breaks in a single strand of the DNA molecule are repaired using the same enzyme systems that are used in Base-Excision Repair (BER).

Double-Strand Breaks (DSBs)

There are two mechanisms by which the cell attempts to repair a complete break in a DNA molecule:

- **Direct joining** of the broken ends. This requires proteins that recognize and bind to the exposed ends and bring them together for ligating. They would prefer to see some complementary nucleotides but can proceed without them so this type of joining is also called **Nonhomologous End-Joining** (**NHEJ**). A protein called Ku is essential for NHEJ. Ku is a heterodimer of the subunits **Ku70** and **Ku80**. Errors in direct joining may be a cause of the various **translocations** that are associated with cancers. Some examples include Burkitt's lymphoma, the Philadelphia chromosome in chronic myelogenous leukemia (CML) and B-cell leukemia.
- **Homologous Recombination (also known as Homology-Directed Repair — HDR).** Here the broken ends are repaired using the information on the intact **sister chromatid** (available in G2 after chromosome duplication), **or** on the **homologous chromosome** (in G1; that is, before each chromosome has been duplicated). This requires searching around in the nucleus for the homolog — a task sufficiently uncertain that G1 cells usually prefer to mend their DSBs by **NHEJ**. **or** on the
- **same chromosome** if there are duplicate copies of the gene on the chromosome oriented in opposite directions (head-to-head or back-to-back).
- Two of the proteins used in homologous recombination are encoded by the genes ***BRCA1*** and ***BRCA2***. Inherited mutations in these genes predispose women to breast and ovarian cancers.

The Generation of Antibody Diversity

Some of the same enzymes used to repair DSBs by **direct joining** are also used to break and reassemble the gene segments used to make

- antibody variable regions; that is, to accomplish **V(D)J joining** — (mice whose Ku80 genes have been knocked out cannot do this);
- different antibody classes; that is, to accomplish **class switching**

Meiosis also involves DSBs

Recombination between homologous chromosomes in meiosis I also involves the formation of DSBs and their repair. So it is not surprising that this process uses the same enzymes.

Meiosis I with the alignment of homologous sequences provides a mechanism for repairing damaged DNA; that is, mutations. in fact, many biologists feel that the main function of sex is to provide this mechanism for maintaining the integrity of the genome.

However, most of the genes on the human Y chromosome have no counterpart on the X chromosome, and thus cannot benefit from this repair mechanism. They seem to solve this problem by having multiple copies of the same gene — oriented in opposite directions. Looping the intervening DNA brings the duplicates together and allowing repair by homologous recombination.

Gene Conversion

If the sequence used as a template for repairing a gene by homologous recombination differs slightly from the gene needing repair; that is, is an allele, the repaired gene will acquire the donor sequence. This **nonreciprocal transfer** of genetic information is called gene conversion.

The donor of the new gene sequence may be:

- the homologous chromosome (during meiosis)
- the sister chromatid (also during meiosis)
- a duplicate of the gene on the same chromosome (during mitosis)

Gene conversion during meiosis alters the normal mendelian ratios. Normally, meiosis in a heterozygous (**A**,**a**) parent will produce gametes or spores in a 1:1 ratio; e.g., 50% **A**; 50% **a**. However, if gene conversion has occurred, other ratios will appear. If, for example, an **A** allele donates its sequence as it repairs a damaged **a** allele, the repaired gene will become **A**, and the ratio will be 75% **A**; 25% **a**.

Cancer Chemotherapy

- The hallmark of all cancers is continuous cell division.
- Each division requires both
  - the replication of the cell's DNA (in S phase) and
  - transcription and translation of many genes needed for continued growth.
- So, any chemical that damages DNA has the potential to inhibit the spread of a cancer.
- Many (but not all) drugs used for cancer therapy do their work by damaging DNA.

The table lists (by trade name as well as generic name) some of the anticancer drugs that specifically target DNA.

6-mercaptopurine	Purinethol®	purine analog. One effect: substitutes for G, inducing abortive MMR and strand breaks
Gemcitabine	Gemzar®	pyrimidine analog substitutes for C blocking strand elongation
Cyclophosphamide	Cytoxan®	alkylating agents; form interstrand and/or intrastrand crosslinks
Melphalan	Alkeran®	
Busulfan	Myleran®	
Chlorambucil	Leukeran®	
Mitomycin	Mutamycin®	
Cisplatin	Platinol®	forms crosslinks
Bleomycin	Blenoxane®	cuts DNA strands between GT or GC
Irinotecan	Camptosar®	inhibit the proper functioning of enzymes (**topoisomerases**) needed to unwind DNA for replication and transcription
Mitoxantrone	Novantrone®	
Doxorubicin	Adriamycin®	
Dactinomycin	Cosmegen®	inserts into the double helix preventing its unwinding

The cancer patient has many other cell types that are also proliferating rapidly, e.g., cells of the intestinal endothelium, bone marrow and hair follicles. Anticancer drugs also damage these — producing many of the unpleasant side effects of "chemo". Agents that damage DNA are themselves carcinogenic, and chemotherapy poses a significant risk of creating a new cancer, often a leukemia.

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5.14: Harlequin Chromosomes



Figure 5.14.2 Immortal Strands

Most eukaryotes have several to many pairs of chromosomes, and we might expect that at metaphase of mitosis the chromosomes would align at the metaphase plate at random so that some containing the immortal DNA strand would go to one pole; the remainder to the other. And this is generally the case. However, there may be some exceptions.

Stem cells divide to produce two daughter cells:

- one that will continue as a stem cell and
- one that will go on to differentiate.

There is evidence that when some types of stem cells divide, for example a subset found in skeletal muscle, the chromatids containing the immortal strand all line up on one side of the metaphase plate and the daughter cell receiving this set is the one that **remains a stem cell**. Although the mechanism by which this occurs is unknown, one can appreciate a potential value to the organism. Errors (mutations) in DNA occur most often during its replication. By keeping the original template in the stem cell population, introduced errors (mutations) disappear when the differentiated cell dies at the end of its useful life. Another possible

advantage of nonrandom segregation of parental vs. newly-synthesized DNA: it may assure that epigenetic alterations of their respective DNA strands are transmitted to the appropriate daughter cells.

(The figure represents a haploid cell with **n** = 2. Each bar represents one strand of the DNA double helix.)

However, other experiments, with other types of stem cells, find that

- only certain chromosomes (e.g., the X and Y in Drosophila male germline stem cells) preferentially segregate the parental chromatids to the cell that will remain a stem cell;
- and for others, the distribution of immortal strands at metaphase is random, and thus the drawing on the right does not reflect what happens in those cases.

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5.15: Metagenomics - Exploring the Microbial World

All the genomes listed on my page Genome Sizes describe the complete genome of a single species. For bacteria and archaeons, this means that the organism was grown in pure culture to provide the DNA for sequencing. But it is now clear that the microbial world contains vast numbers of both groups that have never been grown in the laboratory and thus have escaped study. Soil, water, and the contents of our large intestine are examples of habitats that teem with unknown microorganisms.

Thanks to the recent development of sequencing machines capable of rapidly (and inexpensively) sequencing huge amounts of DNA, it is now practical to sequence the DNA extracted from complex microbial ecosystems like that found in a soil sample. Several different approaches are used, but all depend on a first step of extracting the microbial DNA from the sample (and separating it from the far more complex DNA of any eukaryotes that may be present).

Assessing Microbial Diversity

The DNA encoding the small subunit (16S) of the ribosomes of both bacteria and archaeons contain some highly conserved regions; that is, regions of identical or almost identical sequence. Using primers that target these regions, one can then produce enough material by the polymerase chain reaction PCR to sequence the entire 16S rRNA gene.

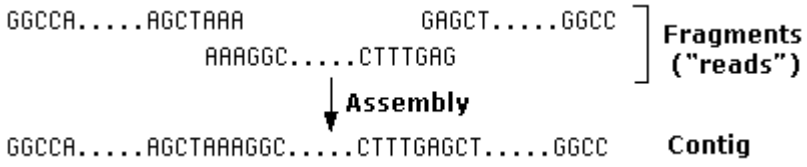
Comparing the various sequences to a database of sequences from known organisms, one can estimate how many different types of microbes are present. Because of the substantial genetic diversity found between "strains" of a single species (e.g., *E. coli* K-12 and *E.coli* O157:H7), closely-related (> 97% identity) 16S rDNA sequences are assigned to a single "**phylotype**" because we cannot be sure whether they belong to separate species or to two strains of the same species. In either case, the collection of 16S rDNA sequences can be arranged to form a phylogenetic tree to show the patterns of relatedness.

Cataloging the Genes in a Microbial Ecosystem

Analyzing the 16S rDNA genes in a sample tells us who is there, but, of course, is not a complete genome and tells us nothing about the other genes present in the various members of the population. This information can be gained by "shotgun" sequencing of the environmental DNA sample.

The Steps:

- Break the DNA in short fragments.
- Insert these into a vector, e.g. a plasmid capable of growing in *E. coli* K-12.
- Expose *E. coli* cells to this random mix and grow the individual bacterial cells into colonies.
- The result: a **library** containing millions of random DNA fragments from the original sample.
- Isolate the plasmids and sequence them. Sequence "reads" average around 100 nucleotides — far shorter than a gene but often enough to move on to the next step.
- Use a powerful computer to attempt to assemble the fragments into a linear sequence of DNA. The computer looks for identical stretches of nucleotides in different fragments and uses the overlap to assemble them into a "**contig**".



- Look (have the computer look) for open reading frames (ORFs) of protein-encoding genes.
- Compare the ORFs with those of known microbes already in databases to see if a function can be deduced.

The sheer diversity of organisms in most microbial ecosystems makes it virtually impossible to find enough contigs to assemble a complete genome for any one organism like those listed in Genome Sizes. What you get instead is a window into the many kinds of genes present in one inhabitant or another of that ecosystem. For example, you may discover genes that encode proteins able to degrade environmental pollutants or genes able to synthesize a new antibiotic.

Finding New Functions in Microbial Populations

Another way of exploiting metagenomics is to look for new functions in the host (e.g. *E. coli*) if it can express the new gene with which it was transformed. For example, screening the library of *E. coli* clones for the ability to resist an antibiotic can reveal genes involved in antibiotic resistance — a worrisome development in recent years.

Some Applications of Metagenomics

- The Sargasso Sea: Metagenomic analysis of the DNA extracted from sea water in the Sargasso Sea revealed the presence of over a thousand different 16S rDNA genes (and thus approximately that number of different species) and over a million protein-encoding genes.
- **The Human Colon**: 0.3 g fecal samples from two healthy humans produced 78 million base pairs of sequence. Each subject produced some 25 thousand open reading frames (ORFs) of which about half could be recognized as already-known bacterial or archaeal genes. Included were genes encoding enzymes for the synthesis of vitamins (e.g., vitamin B_1), amino acids, and enzymes for the digestion of complex polysaccharides in our diet which would otherwise be indigestible. Perhaps as much as 10% of the energy we extract from our food is made available to us by the activity of these microorganisms.
- **Acid Mine Drainage**: Metagenomic analysis of the acidic water (pH ~0.5) flowing from an abandoned metal mine in California revealed a much simpler ecosystem than those described above: only 3 species of bacteria and 2 of archaea. With such limited diversity, it was possible to assemble almost-complete genomes for two of these organisms.
- **A South African Gold Mine:** Simpler still was the ecosystem found in water 2.8 km (1.7 miles) down in a gold mine. Only one organism turned up: an autotrophic bacterium capable of extracting energy from inorganic substances in its environment and synthesizing all the molecules needed for its life from them.

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UNIT 6: GENE EXPRESSION

6.1: One Gene - One Enzyme Theory

6.2: The Transcription of DNA into RNA

6.3: Genetic Code

6.4: The Translation of RNA into Proteins

6.5: RNA Editing

6.6: Expressed Sequence Tags

6.7: Ribosomal RNA (rRNA) Gene Cluster

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6.1: One Gene - One Enzyme Theory

Neurospora crassa

Neurospora crassa is an ascomycete, the red bread mold. Like all fungi, it reproduces by spores. It produces two kinds of spores:

- **Conidia** are spores produced by asexual reproduction. Mitosis of the haploid nuclei of the active, growing fungus generates the conidia.
- **Ascospores**, on the other hand, are formed following sexual reproduction. If two different mating types ("sexes") are allowed to grow together, they will fuse to form a diploid zygote. Meiosis of this zygote then gives rise to the haploid ascospores.

Neurospora is particularly well suited for genetic studies because

- It can be grown quickly on simple culture medium.
- It spends most of its life cycle in the haploid condition so any recessive mutations will show up in its phenotype.
- When the diploid zygote undergoes meiosis, the nuclei produced by
  - Meiosis I, followed by
  - Meiosis II, followed by
  - one mitotic division

  are confined to a narrow tube, the **ascus**.
- Because the nuclei cannot slip past one another, if
  - the zygote nucleus is heterozygous for a gene (shown here as **a** and **A**) and
  - no crossing over near that locus occurs during meiosis I,

  the ascus will finally have four spores at one end containing one allele and four spores at the other end containing the other allele.

The One Gene - One Enzyme Theory

Sucrose, a few salts, and one vitamin — biotin — provide the nutrients that Neurospora needs to synthesize all the macromolecules of its cells.

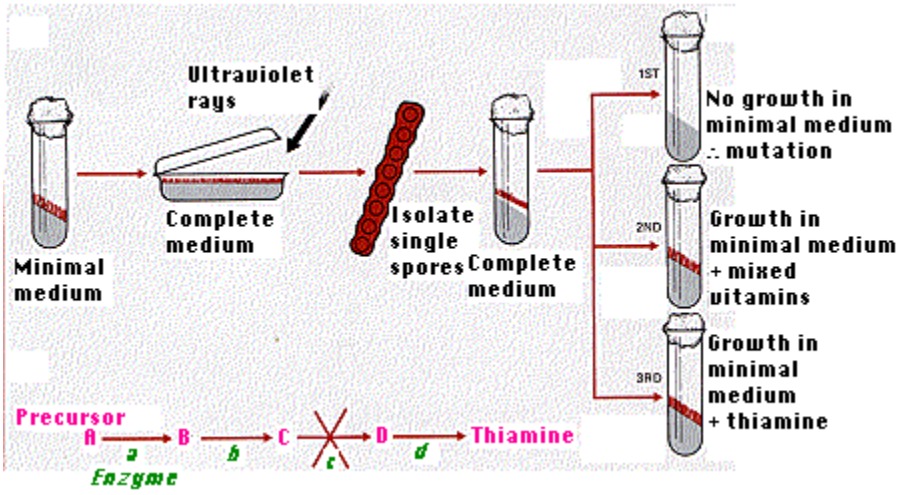


Figure 6.1.1 Beadle - Tatum Experiment on Neurospora

Geneticists George W. Beadle and E. L. Tatum exposed some of the conidia of one mating type of Neurospora to ultraviolet rays in order to induce mutations.

- Then individual irradiated spores were allowed to germinate on a "complete" medium; that is, one enriched with various vitamins and amino acids.

- Once each had developed a mycelium, it was allowed to mate with the other mating type.
- The ascospores produced were dissected out **individually** and each one placed on **complete** medium.
- After growth had occurred, portions of each culture were subcultured on **minimal medium**.
- Sometimes growth continued; sometimes it didn't.
- When it did not ("1st" in the figure) , the particular strain was then supplied with a mixture of vitamins, amino acids, etc. until growth did occur ("2nd ").
- Eventually each mutated strain was found to have acquired a need for **one** nutrient; in the example illustrated here, the vitamin thiamine ("3rd").

Beadle and Tatum reasoned that radiation had caused a gene that permits the synthesis of thiamine from the simple ingredients in minimal medium to mutate to an allele that does not. The synthesis of thiamine from sucrose requires a number of chemical reactions, each one catalyzed by a specific enzyme.

By adding, one at a time, the different precursors of thiamine to the medium in which their mutant mold was placed, they were able to narrow down the defect to the absence of a single enzyme.

- If they added to the minimal medium any precursor further along in the process, growth occurred.
- Any precursor before the blocked step could not support growth.

Thus, in this example, the conversion of precursor **C** to precursor **D** was blocked because of the absence of the needed enzyme (***c***).

This led them to postulate the **one gene - one enzyme** theory: each gene in an organism controls the production of a specific enzyme. It is these enzymes that catalyze the reactions that lead to the phenotype of the organism.

Today, we know that, in fact, not only enzymes, but all the other proteins from which the organism is built are encoded by genes.

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6.2: The Transcription of DNA into RNA

The majority of genes are expressed as the proteins they encode. The process occurs in two steps:

- **Transcription = DNA → RNA**
- **Translation = RNA → protein**

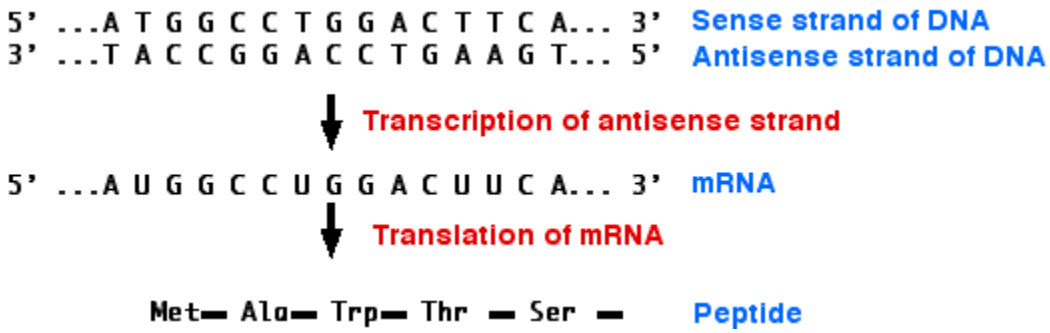


Figure 6.2.1: Here is an overview of the central dogma.

Gene Transcription: DNA → RNA

DNA serves as the template for the synthesis of RNA much as it does for its own replication.

The Steps of Transcription

- Some 50 different protein **transcription factors** bind to **promoter** sites, usually on the 5′ side of the gene to be transcribed.
- An enzyme, an **RNA polymerase**, binds to the complex of transcription factors.
- Working together, they open the DNA double helix.
- The RNA polymerase proceeds to **read** one strand moving in it's 3'→ 5' direction.
- In eukaryotes, this requires — at least for protein-encoding genes — that the nucleosomes in front of the advancing RNA polymerase (Pol II) be removed. A complex of proteins is responsible for this. The same complex replaces the nucleosomes after the DNA has been transcribed and Pol II has moved on.
- As the RNA polymerase travels along the DNA strand, it assembles **ribo**nucleotides (supplied as triphosphates, e.g., ATP) into a strand of RNA.
- Each ribonucleotide is inserted into the growing RNA strand following the rules of base pairing. Thus for each C encountered on the DNA strand, a G is inserted in the RNA; for each G, a C; and for each T, an A. However, each A on the DNA guides the insertion of the pyrimidine uracil (**U**, from uridine triphosphate, UTP). There is no T in RNA.

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- Synthesis of the RNA proceeds in the 5′ → 3′ direction.
- As each nucleoside triphosphate is brought in to add to the 3′ end of the growing strand, the two terminal phosphates are removed.
- When transcription is complete, the transcript is released from the polymerase and, shortly thereafter, the polymerase is released from the DNA.

Note that at any place in a DNA molecule, either strand may be serving as the template; that is, some genes "run" one way, some the other (and in a few remarkable cases, the same segment of double helix contains genetic information on both strands!). In all

cases, however, RNA polymerase transcribes the DNA strand in its 3'→ 5' direction.

A report in the 4 January 2001 issue of Nature shows that RNA polymerase actually tracks around the double helix of DNA. In vitro, at least, when RNA polymerase is immobilized, it spins the DNA molecule around and around as it moves along the molecule. Whether it is the polymerase or the DNA that does the spinning in vivo remains to be determined.

Types of RNA

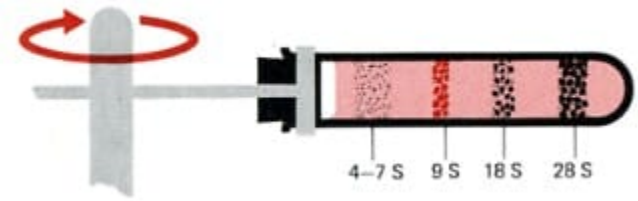


Figure 6.2.1 Sedimentation pattern of RNA

Sedimentation pattern produced by high-speed centrifugation of RNA extracted from the precursors of rabbit red blood cells. The discrete bands represent particular classes of RNA. The transfer RNAs band at about 4S. The ribosomal RNAs of eukaryotes sediment at 5S, 5.8S, 18S, and 28S. (The larger the sedimentation unit, S, the larger the molecule — but not proportionally.) The RNA forming the band at 9S is the messenger RNA for the synthesis of hemoglobin, the major protein synthesized in these cells. In most types of cells, the messenger RNAs are extremely heterogenous, with small amounts distributed from 6S to 25S.

Several types of RNA are synthesized in the nucleus of eukaryotic cells.

- messenger RNA (**mRNA**)
- **ribosomal RNA** (**rRNA**)
- **transfer RNA** (**tRNA**)
- **small nuclear RNA** (**snRNA**)
- **small nucleolar RNA** (**snoRNA**)
- **microRNA** (**miRNA**). These are tiny (~22 nucleotides) RNA molecules that regulate the expression of messenger RNA (mRNA) molecules.
- **long non-coding RNA** (**lncRNA**)

Messenger RNA (mRNA)

Messenger RNA will be **translated** into a polypeptide. Messenger RNA comes in a wide range of sizes reflecting the size of the polypeptide it encodes. Most cells produce small amounts of thousands of different mRNA molecules, each to be translated into a peptide needed by the cell. Many mRNAs are common to most cells, encoding "housekeeping" proteins needed by all cells (e.g., the enzymes of glycolysis). Other mRNAs are specific for only certain types of cells. These encode proteins needed for the function of that particular cell (e.g., the mRNA for hemoglobin in the precursors of red blood cells).

Ribosomal RNA (rRNA)

This will be used in the building of ribosomes: machinery for synthesizing proteins by translating mRNA. There are 4 kinds. In eukaryotes, these are

- 18S rRNA. One of these molecules, along with some 30 different protein molecules, is used to make the **small subunit** of the ribosome.
- **28S, 5.8S, and 5S rRNA**. One each of these molecules, along with some 45 different proteins, are used to make the **large subunit** of the ribosome.

The S number given each type of rRNA reflects the rate at which the molecules sediment in the ultracentrifuge. The larger the number, the larger the molecule (but not proportionally). The 28S, 18S, and 5.8S molecules are produced by the processing of

a single primary transcript from a cluster of identical copies of a single gene. The 5S molecules are produced from a different cluster of identical genes.

Transfer RNA (tRNA)

These are the RNA molecules that carry amino acids to the growing polypeptide. There are some 32 different kinds of tRNA in a typical eukaryotic cell.

- Each is the product of a separate gene.
- They are small (~4S), containing 73-93 nucleotides.
- Many of the bases in the chain pair with each other forming sections of double helix.
- The unpaired regions form 3 loops.
- Each kind of tRNA carries (at its 3′ end) one of the 20 **amino acids** (thus most amino acids have more than one tRNA responsible for them).
- At one loop, 3 unpaired bases form an **anticodon**.
- Base pairing between the anticodon and the complementary codon on a mRNA molecule brings the correct amino acid into the growing polypeptide chain.

Small Nuclear RNA (snRNA)

DNA transcription of the genes for mRNA, rRNA, and tRNA produces large precursor molecules (**"primary transcripts"**) that must be processed within the nucleus to produce the functional molecules for export to the cytosol. Some of these processing steps are mediated by snRNAs.

Approximately a dozen different genes for snRNAs, each present in multiple copies, have been identified. The snRNAs have various roles in the processing of the other classes of RNA. For example, several snRNAs are part of the **spliceosomes** that participate in converting pre-mRNA into mRNA by excising the introns and splicing the exons.

Small Nucleolar RNA (snoRNA)

As the name suggests, these small (60–300 nucleotides) RNAs are found in the nucleolus where they are responsible for several functions:

- Some participate in making ribosomes by helping to cut up the large RNA precursor of the 28S, 18S, and 5.8S molecules.
- Others chemically modify many of the nucleotides in rRNA, tRNA, and snRNA molecules, e.g., by adding methyl groups to ribose.
- Some have been implicated in the alternative splicing of pre-mRNA to different forms of mature mRNA.
- One snoRNA serves as the template for the synthesis of telomeres.

In vertebrates, the snoRNAs are made from **introns** removed during RNA processing.

MicroRNAs (miRNAs)

MicroRNAs" ("miRNAs") are single-stranded RNA molecules containing about 22 nucleotides and are about the same size as siRNAs. MicroRNAs are found in all animals (humans generate some 1000 miRNAs) and plants but not in fungi. They contain 19–25 nucleotide. They are

- encoded in the genome
  - some by stand-alone genes (that may encode several miRNAs)
  - some by portions of an intron of the gene whose mRNA they will regulate.
- may be expressed in
  - only certain cell types and
  - at only certain times in the differentiation of a particular cell type.

While direct evidence of the function of many of these newly-discovered gene products remains to be discovered, they regulate gene expression by regulating messenger RNA (mRNA), either

- destroying the mRNA when the sequences match exactly (the usual situation in plants) or
- repressing its **translation** when the sequences are only a partial match.

MicroRNAs have two traits ideally suited for this:

- Being so small, they can be rapidly transcribed from their genes.
- They do not need to be translated into a protein product to act.

MicroRNAs regulate (repress) expression of genes in mammals as well. Genome analysis has revealed thousands of human genes whose transcripts (mRNAs) contain sequences to which one or more of our miRNAs might bind. Probably each miRNA can bind to as many as 200 different mRNA targets while each mRNA has binding sites for multiple miRNAs. Such a system provides many opportunities for coordinated mRNA translation

Long Non-coding RNA (lncRNA)

Only messenger RNA encodes polypeptides. All the other classes of RNA are thus called non-coding RNA. In addition to the rRNAs, snRNAs, and snoRNAs, there is a large (more than 10,000 in humans), heterogenous collection of transcripts longer than 200 nucleotides that are classified as lncRNAs. The function, if any, of most of these remains to be discovered.

However, some lncRNAs have been found to participate in the regulation of such diverse activities as splicing, translation, imprinting, and transcription. Two examples:

- XIST RNA, which contains thousands of nucleotides, inactivates one of the two X chromosomes in female vertebrates.
- Some lncRNAs participate in bringing the enhancer and promoter regions of genes close together ("looping") to regulate gene transcription.

While much remains to be learned about their functions, taken together non-coding RNAs probably account for three-quarters of the transcription going on in the nucleus.

The RNA polymerases

The RNA polymerases are huge multi-subunit protein complexes. Three kinds are found in eukaryotes.

- RNA polymerase I (**Pol I**). It transcribes the **rRNA** genes for the precursor of the 28S, 18S, and 5.8S molecules (and is the busiest of the RNA polymerases).
- RNA polymerase II (**Pol II**; also known as **RNAP II**). It transcribes protein-encoding genes into **mRNA** (and also the **snRNA** genes).
- RNA polymerase III (**Pol III**). It transcribes the 5S rRNA genes and all the **tRNA** genes.

RNA Processing: pre-mRNA → mRNA

All the primary transcripts produced in the nucleus must undergo processing steps to produce functional RNA molecules for export to the cytosol. We shall confine ourselves to a view of the steps as they occur in the processing of pre-mRNA to mRNA.

Most eukaryotic genes are split into segments. In decoding the open reading frame of a gene for a known protein, one usually encounters periodic stretches of DNA calling for amino acids that do not occur in the actual protein product of that gene. Such stretches of DNA, which get transcribed into RNA but not translated into protein, are called **introns**. Those stretches of DNA that do code for amino acids in the protein are called **exons**. Examples:

- The gene for one type of collagen found in chickens is split into 52 separate exons.
- The gene for **dystrophin**, which is mutated in boys with muscular dystrophy, has 79 exons.
- Even the genes for rRNA and tRNA are split by introns.
- The human genome is estimated to contain some 180,000 exons. With a current estimate of 21,000 genes, the average exon content of our genes is about 9.

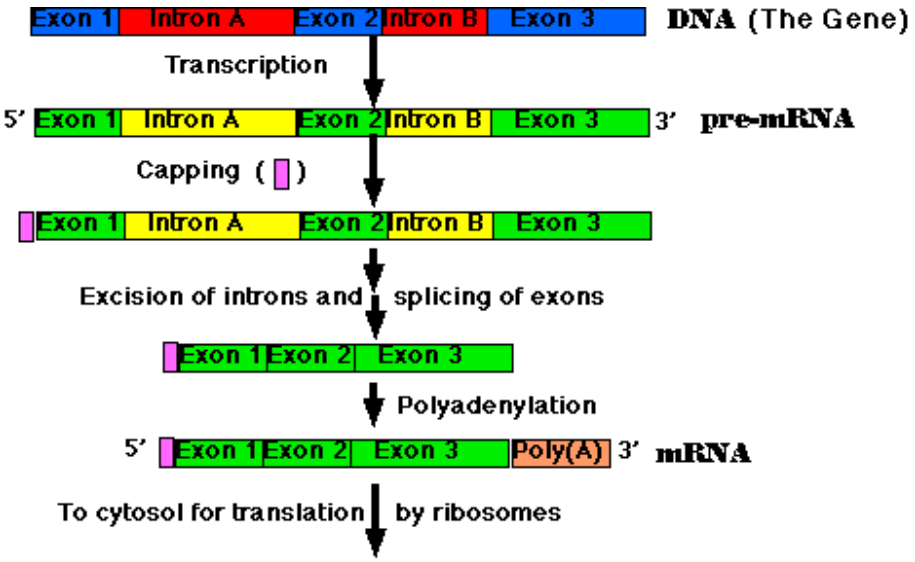


Figure 6.2.2 RNA Processing

- Synthesis of the **cap**. This is a modified guanine (G) which is attached to the 5′ end of the pre-mRNA as it emerges from RNA polymerase II (Pol II). The cap
- protects the RNA from being degraded by enzymes that degrade RNA from the 5′ end;
- serves as an assembly point for the proteins needed to recruit the small subunit of the ribosome to begin translation.

- Step-by-step removal of **introns** present in the pre-mRNA and splicing of the remaining **exons**. This step takes place as the pre-mRNA continues to emerge from Pol II.
- Synthesis of the **poly(A) tail**. This is a stretch of adenine (A) nucleotides. When a special poly(A) attachment site in the pre-mRNA emerges from Pol II, the transcript is cut there, and the poly(A) tail is attached to the exposed 3′ end. This completes the mRNA molecule, which is now ready for export to the cytosol. (The remainder of the transcript is degraded, and the RNA polymerase leaves the DNA.)

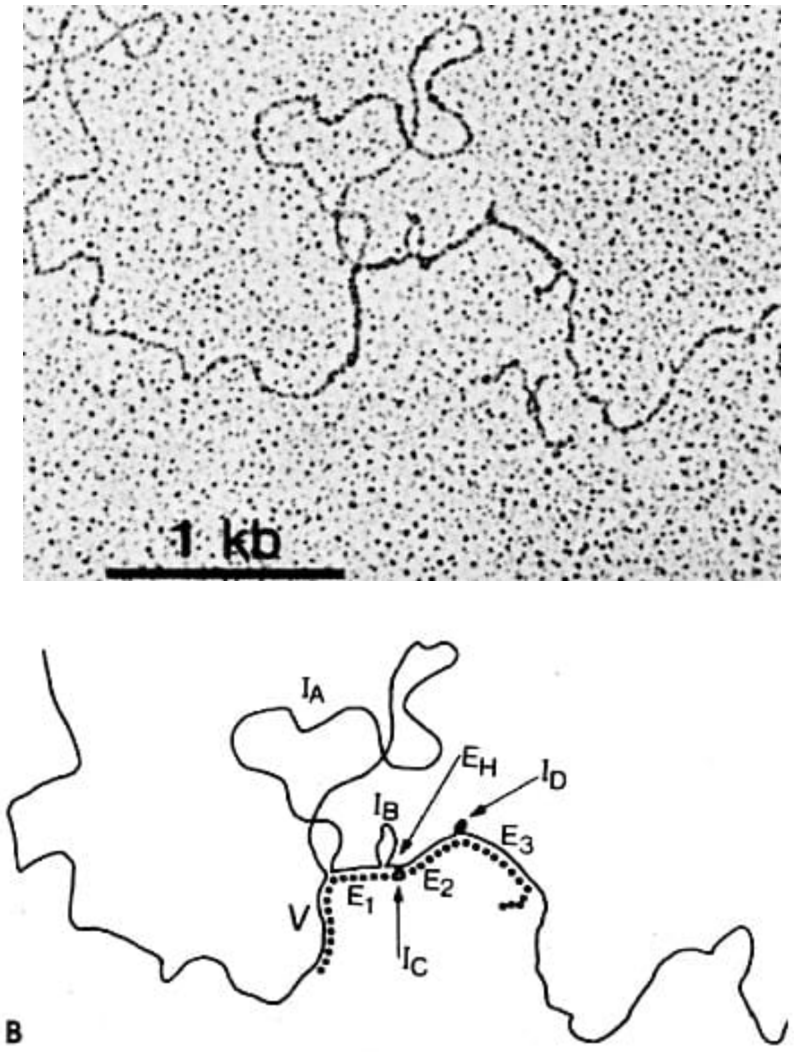


Figure 6.2.3 mRNA-DNA hybrid molecule

The above image is an electron micrograph of a mRNA-DNA hybrid molecule formed by mixing the messenger RNA (mRNA) from a clone of antibody-secreting cells with single-stranded DNA from the same kind of cells. The bar represents the length of 1000 bases. The lower diagram is an interpretation of the micrograph. The solid line represents the DNA; the dotted line the mRNA. The loops (I_A, I_B, etc.) represent the introns that separate the exons encoding the domains of an antibody heavy chain:

- V = variable region
- E_1 = first constant region (C_H1) domain
- E_H = hinge region
- E_2 and E_3 = the nucleotides encoding the two C-terminal domains (C_H2 and C_H3)

The unhybridized portion of the mRNA is its poly(A) tail.

Alternative Splicing

The processing of pre-mRNA for many proteins proceeds along various paths in different cells or under different conditions. For example, early in the differentiation of a B cell (a lymphocyte that synthesizes an antibody) the cell first uses an exon that encodes a transmembrane domain that causes the molecule to be retained at the cell surface. Later, the B cell switches to using a different exon whose domain enables the protein to be secreted from the cell as a circulating antibody molecule.

Alternative splicing provides a mechanism for producing a wide variety of proteins from a small number of genes. While we humans may turn out to have only some 20 thousand genes, we probably make at least 10 times that number of different proteins. It is now estimated that 92–94% of our genes produce pre-mRNAs that are alternatively-spliced. There is evidence that the pattern of alternative splicing differs consistently in different tissues and so must be regulated. But whether all the products are functional or that many are simply the outcome of an error-prone process remains to be seen.

Alternative splicing not only provides different proteins from a single gene but also different 3' UTRs and 5' UTRs. Although not translated into protein, these **un**t**r**anslated **r**egion**s** contain signals that, for example, dictate where in the cell that protein will accumulate. Two examples:

- The 3' UTR of the *bicoid* gene in Drosophila directs the mRNA to the anterior of the embryo
- the same region in the *VegT* gene of Xenopus directs its mRNA to the vegetal pole of the embryo

One of the most dramatic examples of alternative splicing is the ***Dscam*** gene in **Drosophila**. This single gene contains some 116 exons of which 17 are retained in the final mRNA. Some exons are always included; others are selected from an array. Theoretically this system is able to produce 38,016 different proteins. And, in fact, over 18,000 different ones have been found in Drosophila hemolymph.

These Dscam proteins are used to establish a unique identity for each neuron. It works like this. Each developing neuron synthesizes a dozen or so Dscam mRNAs out of the thousands of possibilities. Which ones are selected appears to be simply a matter of chance, but because of the great number of possibilities, each neuron will most likely end up with a unique set of a dozen or so Dscam proteins. As each developing neuron in the central nervous system sprouts dendrites and an axon, these express its unique collection of Dscam proteins. If the various extensions of a single neuron should meet each other in the tangled web that is the hallmark of nervous tissue, they are repelled. In this way, thousands of different neurons can coexist in intimate contact without the danger of nonfunctional contacts between the various extensions of the same neuron.

neuron should encounter each other, they avoid establishing a synapse by the repulsion mediated by their identical collection of protocadherins. In this way, thousands of different neurons can coexist in intimate contact without the danger of nonfunctional contacts between the various extensions of the same neuron.

Whether a particular segment of RNA will be retained as an exon or excised as an intron can vary under different circumstances, such as

- what type of cell the gene is in
- what stage of differentiation that cell is passing through
- what extracellular signals that cell is receiving.

Clearly the switching to an alternate splicing pathway must be closely regulated.

Trans-splicing

Most genes are transcribed and their transcripts processed as described above. RNA polymerase travels down a single strand of a single gene locus to form pre-mRNA that is processed (including removal of introns) to form the mature mRNA. But there are exceptions. A number of cases have been found where two different precursor transcripts have been spliced together to form the final RNA molecule. The phenomenon is called *trans*-splicing.

Examples: synthesis of a single RNA molecule by splicing together transcripts from loci

- located far apart on the same chromosome or
- on opposite strands of the same gene locus or
- that are the two alleles of the gene on their separate (homologous) chromosomes.

The biological importance of these *trans*-spliced transcripts is still unknown for most of them.

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6.3: Genetic Code

The genetic code consists of 64 triplets of nucleotides. These triplets are called **codons**.With three exceptions, each codon encodes for one of the 20 amino acids used in the synthesis of proteins. That produces some redundancy in the code: most of the amino acids being encoded by more than one codon.

One codon, **AUG** serves two related functions:

- It signals the start of translation
- It codes for the incorporation of the amino acid methionine (Met) into the growing polypeptide chain

The genetic code can be expressed as either RNA codons or DNA codons. RNA codons occur in messenger RNA (**mRNA**) and are the codons that are actually "read" during the synthesis of polypeptides (the process called **translation**). But each mRNA molecule acquires its sequence of nucleotides by **transcription** from the corresponding gene. Because DNA sequencing has become so rapid and because most genes are now being discovered at the level of DNA before they are discovered as mRNA or as a protein product, it is extremely useful to have a table of codons expressed as DNA. So here are both.

Note that for each table, the left-hand column gives the first nucleotide of the codon, the 4 middle columns give the second nucleotide, and the last column gives the third nucleotide.

The RNA Codons

Second nucleotide

	U	**C**	**A**	**G**	
U	UUU **Phenylalanine** (Phe)	UCU **Serine** (Ser)	UAU **Tyrosine** (Tyr)	UGU **Cysteine** (Cys)	**U**
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	**C**
	UUA **Leucine** (Leu)	UCA Ser	UAA **STOP**	UGA **STOP**	**A**
	UUG Leu	UCG Ser	UAG **STOP**	UGG **Tryptophan** (Trp)	**G**
C	CUU **Leucine** (Leu)	CCU **Proline** (Pro)	CAU **Histidine** (His)	CGU **Arginine** (Arg)	**U**
	CUC Leu	CCC Pro	CAC His	CGC Arg	**C**
	CUA Leu	CCA Pro	CAA **Glutamine** (Gln)	CGA Arg	**A**
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	**G**
A	AUU **Isoleucine** (Ile)	ACU **Threonine** (Thr)	AAU **Asparagine** (Asn)	AGU **Serine** (Ser)	**U**
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	**C**
	AUA Ile	ACA Thr	AAA **Lysine** (Lys)	AGA **Arginine** (Arg)	**A**
	AUG **Methionine** (Met) or **START**	ACG Thr	AAG Lys	AGG Arg	**G**
G	GUU **Valine** Val	GCU **Alanine** (Ala)	GAU **Aspartic acid** (Asp)	GGU **Glycine** (Gly)	**U**
	GUC (Val)	GCC Ala	GAC Asp	GGC Gly	**C**
	GUA Val	GCA Ala	GAA **Glutamic acid** (Glu)	GGA Gly	**A**
	GUG Val	GCG Ala	GAG Glu	GGG Gly	**G**

The DNA Codons

These are the codons as they are read on the sense (5' to 3') strand of DNA. Except that the nucleotide thymidine (**T**) is found in place of uridine (**U**), they read the same as RNA codons. However, mRNA is actually synthesized using the antisense strand of

DNA (3' to 5') as the template.

This table could well be called the Rosetta Stone of life.

The Genetic Code (DNA)

TTT	Phe		TCT	Ser		TAT	Tyr		TGT	Cys
TTC	Phe		TCC	Ser		TAC	Tyr		TGC	Cys
TTA	Leu		TCA	Ser		TAA	STOP		TGA	STOP
TTG	Leu		TCG	Ser		TAG	STOP		TGG	Trp
CTT	Leu		CCT	Pro		CAT	His		CGT	Arg
CTC	Leu		CCC	Pro		CAC	His		CGC	Arg
CTA	Leu		CCA	Pro		CAA	Gln		CGA	Arg
CTG	Leu		CCG	Pro		CAG	Gln		CGG	Arg
ATT	Ile		ACT	Thr		AAT	Asn		AGT	Ser
ATC	Ile		ACC	Thr		AAC	Asn		AGC	Ser
ATA	Ile		ACA	Thr		AAA	Lys		AGA	Arg
ATG	Met*		ACG	Thr		AAG	Lys		AGG	Arg
GTT	Val		GCT	Ala		GAT	Asp		GGT	Gly
GTC	Val		GCC	Ala		GAC	Asp		GGC	Gly
GTA	Val		GCA	Ala		GAA	Glu		GGA	Gly
GTG	Val		GCG	Ala		GAG	Glu		GGG	Gly

*When within gene; at beginning of gene, ATG signals where translation of the RNA will begin.

Codon Bias

All but two of the amino acids (Met and Trp) can be encoded by from 2 to 6 different codons. However, the genome of most organisms reveals that certain codons are preferred over others. In humans, for example, alanine is encoded by GCC four times as often as by GCG. This probably reflects a greater translation efficiency by the translation apparatus (e.g., ribosomes) for certain codons over their synonyms.

Exceptions to the Code

The genetic code is **almost** universal. The same codons are assigned to the same amino acids and to the same START and STOP signals in the vast majority of genes in animals, plants, and microorganisms. However, some exceptions have been found. Most of these involve assigning one or two of the three STOP codons to an amino acid instead.

Mitochondrial genes

When mitochondrial mRNA from animals or microorganisms (but not from plants) is placed in a test tube with the cytosolic protein-synthesizing machinery (amino acids, enzymes, tRNAs, ribosomes) it fails to be translated into a protein.One of the reasons is because these mitochondria use UGA to encode tryptophan (Trp) rather than as a chain terminator. When translated by cytosolic machinery, synthesis stops where Trp should have been inserted. In addition, most animal mitochondria use AUA for methionine not isoleucine and all vertebrate mitochondria use AGA and AGG as chain terminators. Yeast mitochondria assign all codons beginning with CU to threonine instead of leucine (which is still encoded by UUA and UUG as it is in cytosolic mRNA).

Plant mitochondria use the universal code, and this has permitted angiosperms to transfer mitochondrial genes to their nucleus with great ease.

Nuclear genes

Violations of the universal code are far rarer for nuclear genes.

A few unicellular eukaryotes have been found that use one or two (of their three) STOP codons for amino acids instead.

Nonstandard Amino Acids

The vast majority of proteins are assembled from the 20 amino acids listed above even though some of these may be chemically altered, e.g. by phosphorylation, at a later time.

However, two cases have been found where an amino acid that is not one of the standard 20 is inserted by a **tRNA** into the growing polypeptide.

- selenocysteine. This amino acid is encoded by UGA. UGA is still used as a chain terminator, but the translation machinery is able to discriminate when a UGA codon should be used for selenocysteine rather than STOP. This codon usage has been found in certain Archaea, eubacteria, and animals (humans synthesize 25 different proteins containing selenium).
- **pyrrolysine**. In several species of Archaea and bacteria, this amino acid is encoded by UAG. How the translation machinery knows when it encounters UAG whether to insert a tRNA with pyrrolysine or to stop translation is not yet known.

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6.4: The Translation of RNA into Proteins

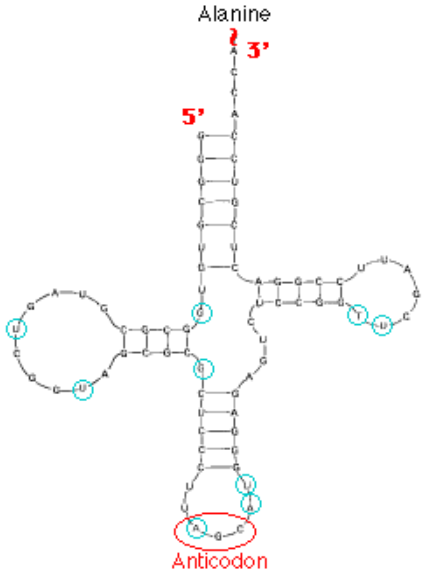


Figure 6.4.1 A**lanine transfer RNA**

This image shows the structure of **alanine transfer RNA** (**tRNA^{ala}**) from yeast. It consists of a single strand of 77 ribonucleotides. The chain is folded on itself, and many of the bases pair with each other forming four helical regions. Loops are formed in the unpaired regions of the chain. (The bases circled in blue have been chemically-modified following synthesis of the molecule.)

At least one kind of tRNA is present for each of the 20 amino acids used in protein synthesis. (Some amino acids employ the services of two or three different tRNAs, so most cells contain as many as 32 different kinds of tRNA.) The amino acid is attached to the appropriate tRNA by an activating enzyme (one of 20 **aminoacyl-tRNA synthetases**) specific for that amino acid as well as for the tRNA assigned to it.

Each kind of tRNA has a sequence of 3 unpaired nucleotides — the **anticodon** — which can bind, following the rules of base pairing, to the complementary triplet of nucleotides — the **codon** — in a **messenger RNA** (**mRNA**) molecule. Just as DNA replication and transcription involve base pairing of nucleotides running in opposite direction, so the reading of codons in mRNA (5' -> 3') requires that the anticodons bind in the opposite direction.

Anticodon: 3' CGA 5'
Codon: 5' GCU 3'

The RNA Codons

Second nucleotide

	U	C	A	G	
U	UUU **Phenylalanine** (Phe)	UCU **Serine** (Ser)	UAU **Tyrosine** (Tyr)	UGU **Cysteine** (Cys)	**U**
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	**C**
	UUA **Leucine** (Leu)	UCA Ser	UAA **STOP**	UGA **STOP**	**A**
	UUG Leu	UCG Ser	UAG **STOP**	UGG **Tryptophan** (Trp)	**G**
C	CUU **Leucine** (Leu)	CCU **Proline** (Pro)	CAU **Histidine** (His)	CGU **Arginine** (Arg)	**U**
	CUC Leu	CCC Pro	CAC His	CGC Arg	**C**

	CUA Leu	CCA Pro	CAA **Glutamine** (Gln)	CGA Arg	**A**
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	**G**
A	AUU **Isoleucine** (Ile)	ACU **Threonine** (Thr)	AAU **Asparagine** (Asn)	AGU **Serine** (Ser)	**U**
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	**C**
	AUA Ile	ACA Thr	AAA **Lysine** (Lys)	AGA **Arginine** (Arg)	**A**
	AUG **Methionine** (Met) or **START**	ACG Thr	AAG Lys	AGG Arg	**G**
G	GUU **Valine** Val	GCU **Alanine** (Ala)	GAU **Aspartic acid** (Asp)	GGU **Glycine** (Gly)	**U**
	GUC (Val)	GCC Ala	GAC Asp	GGC Gly	**C**
	GUA Val	GCA Ala	GAA **Glutamic acid** (Glu)	GGA Gly	**A**
	GUG Val	GCG Ala	GAG Glu	GGG Gly	**G**

Note:

- Most of the amino acids are encoded by synonymous codons that differ in the third position of the codon.
- In some cases, a single tRNA can recognize two or more of these synonymous codons.
- Example: phenylalanine tRNA with the anticodon 3' AAG 5' recognizes not only UUC but also UUU.
- The violation of the usual rules of base pairing at the third nucleotide of a codon is called "wobble"
- The codon **AUG** serves two related functions
  - It begins every message; that is, it signals the **start of translation** placing the amino acid **methionine** at the amino terminal of the polypeptide to be synthesized.
  - When it occurs within a message, it guides the incorporation of methionine.
- Three codons, **UAA**, **UAG**, and **UGA**, act as signals to terminate translation. They are called **STOP** codons.

The Steps of Translation

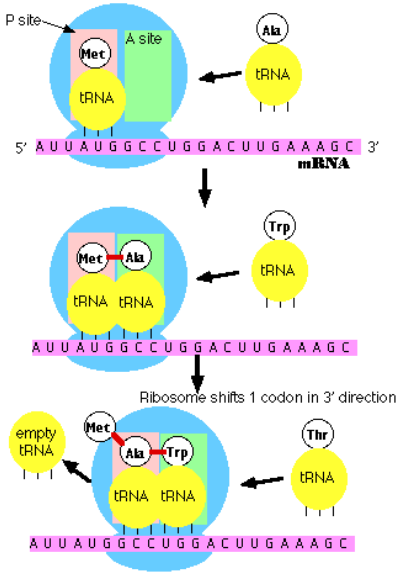


Figure 6.4.2 Translation

Initiation

- The **small subunit** of the ribosome binds to a site "upstream" (on the 5' side) of the start of the message.
- It proceeds downstream (5' -> 3') until it encounters the start codon **AUG**. (The region between the mRNA cap and the AUG is known as the 5'-untranslated region [**5'-UTR**].)
- Here it is joined by the **large subunit** and a special **initiator tRNA**.

- The initiator tRNA binds to the **P site** (shown in pink) on the ribosome.
- In eukaryotes, initiator tRNA carries **methionine** (Met). (Bacteria use a modified methionine designated **fMet**.)

Elongation

- An **aminoacyl-tRNA** (a tRNA covalently bound to its amino acid) able to base pair with the next codon on the mRNA arrives at the **A site** (green) associated with:
  - an **elongation factor** (called EF-Tu in bacteria; EF-1 in eukaryotes)
  - GTP (the source of the needed energy)
- The preceding amino acid (Met at the start of translation) is covalently linked to the incoming amino acid with a peptide bond (shown in red).
- The initiator tRNA is released from the P site.
- The ribosome moves one codon downstream.
- This shifts the more recently-arrived tRNA, with its attached peptide, to the P site and opens the A site for the arrival of a new aminoacyl-tRNA.
- This last step is promoted by another protein **elongation factor** (called EF-G in bacteria; EF-2 in eukaryotes) and the energy of another molecule of **GTP**.

Note: the initiator tRNA is the only member of the tRNA family that can bind directly to the P site. The P site is so-named because, with the exception of initiator tRNA, it binds only to a **p**eptidyl-tRNA molecule; that is, a tRNA with the growing peptide attached.

The A site is so-named because it binds only to the incoming **a**minoacyl-tRNA; that is the tRNA bringing the next amino acid. So, for example, the tRNA that brings Met into the interior of the polypeptide can bind only to the A site.

Termination

- The end of translation occurs when the ribosome reaches one or more **STOP** codons (**UAA**, **UAG**, **UGA**). (The nucleotides from this point to the poly(A) tail make up the 3'-untranslated region [**3'-UTR**] of the mRNA.)
- There are no tRNA molecules with anticodons for STOP codons.
- However, protein **release factors** recognize these codons when they arrive at the A site.
- Binding of these proteins —along with a molecule of **GTP** — releases the polypeptide from the ribosome.
- The ribosome splits into its subunits, which can later be reassembled for another round of protein synthesis.

Polysomes

A single mRNA molecule usually has many ribosomes traveling along it, in various stages of synthesizing the protein. This complex is called a **polysome.**

Codon Bias

All but two of the amino acids (Met and Trp) can be encoded by from 2 to 6 different codons. However, the genome of most organisms reveals that certain codons are preferred over others. In humans, for example, alanine is encoded by GCC four times as often as by GCG. This probably reflects a greater translation efficiency by the translation apparatus for certain codons over their synonyms.

- At the start of translation, two or more of a set of synonymous codons (e.g., the 6 codons that incorporate leucine in the growing protein) are used **alternately**. The need to locate first one and then another tRNA for that amino acid slows down the rate of translation.
  - This may aid in keeping ribosomes from bumping into each other on the polysome.
  - It may also provide more time for the nascent protein to begin to fold correctly as it emerges from the ribosome.
- Once translation is well underway (after 30–50 amino acids have been added), one particular codon tends to be chosen each time its amino acid is called for. Presumably this now increases the efficiency (speed) of translation.
- Most organisms have more than the 61 genes needed to encode a tRNA for each of the 61 codons (we have 270 tRNA genes). The presence of multiple genes for tRNAs with an **identical anticodon** increases the concentration of tRNAs able to bind a particular codon. Messenger RNAs — especially those of active genes — tend to favor codons that correspond to abundant tRNAs carrying the anticodon.

Codon bias even extends to pairs of codons: wherever a human protein contains the amino acids Ala-Glu, the gene encoding those amino acids is seven times as likely to use the codons GCAGAG rather than the synonymous GCCGAA. Codon bias is exploited by the biotechnology industry to improve the yield of the desired product. The ability to manipulate codon bias may also usher in a era of safer vaccines.

Quality Control

Defective mRNA molecules can be produced by mutations in the gene as well as errors introduced during transcription (albeit at a remarkably low rate). In addition to producing mRNAs with incorrect codons for amino acids, these errors can produce mRNA molecules that have

- Premature Termination Codons (PTCs); that is, the introduction of a STOP codon before the normal end of the message. Translation of these mRNAs produces a truncated protein that is probably ineffective and may be harmful. The problem can sometimes be solved by Nonsense-Mediated mRNA Decay (NMD).
- **no** STOP codon. These produce "nonstop" transcripts. The problem can be solved by Nonstop mRNA Decay.

Nonsense-Mediated mRNA Decay (NMD)

Premature termination codons (PTCs) may be generated by "nonsense" mutations, frameshifts, and RNA processing (intron removal) errors. They are also an inevitable consequence of creating antigen receptors on B cells and T cells.

Mechanisms

- During RNA processing within the nucleus, protein complexes are added at each spot where adjacent exons are spliced together. (These are important signals for exporting the mRNA to the cytoplasm.)
- In the cytoplasm, as the ribosome moves down the mRNA, these complexes are removed (and sent back to the nucleus for reuse).
- If the ribosome encounters a premature termination codon, the final exon-exon tag(s) are not removed, and this marks the defective mRNA for destruction (in P bodies).

Mutations that introduce premature termination codons are responsible for some cases of such inherited human diseases as cystic fibrosis and Duchenne muscular dystrophy (DMD).

A drug, designated **PTC124** or ataluren, causes the ribosome to skip over PTCs while still enabling normal termination of translation. PTC124 has shown promise in animal models of cystic fibrosis and DMD and phase II clinical trials are now being conducted on humans.

Nonstop mRNA Decay

Nonstop transcripts occur when there is no STOP codon in the message. As a result the ribosome is unable to recruit the **release factors** needed to leave the mRNA. Nonstop transcripts are formed during RNA processing, e.g., by having the poly(A) tail put on before the STOP codon is reached.

Mechanisms

Eukaryotes and bacteria handle the problem of no STOP codon differently.

- In **eukaryotes**, when the ribosome stalls at the end of the poly(A) tail, **proteins** are recruited to release the ribosome for reuse and to degrade the faulty message.
- In **bacteria**, a special RNA molecule — called **tmRNA** saves the day. It is called tmRNA because it has the properties of both a **t**ransfer RNA and a **m**essenger RNA. The transfer part adds alanine to the A site on the ribosome. The ribosome then moves on to the messenger part which encodes 10 amino acids that target the molecule for destruction (and releases the ribosome for reuse).

Regulation of Translation

The expression of most genes is controlled at the level of their transcription. Transcription factors (proteins) bind to promoters and enhancers turning on (or off) the genes they control. However, gene expression can also be controlled at the level of translation.

By General RNA-Degradation Machinery

P bodies

The cytosol of eukaryotes contains protein complexes that compete with ribosomes for access to mRNAs. As these increase their activity, they sequester mRNAs in larger aggregates called **P bodies** (for "processing bodies", but this processing should not be confused with the processing of pre-mRNA to mature mRNA that occurs in the nucleus).

These protein complexes break down the mRNA by

- removing its "cap"
- removing its poly(A) tail
- degrading the remaining message (nibbling away in the 5' -> 3' direction)

What controls the dynamic balance between ribosomes and P bodies for access to mRNAs remains to be learned. But this mechanism provides for

- destruction of "bad" mRNAs (e.g., those with premature STOP codons
- turnover of mRNAs thus increasing the flexibility of gene expression in the cell

Exosomes

These are hollow macromolecular complexes with two openings. They take in unfolded RNA molecules and degrade them in the 3' -> 5' direction. (In neither structure nor function do these exosomes resemble the exosomes involved in antigen presentation that unfortunately share the same name.)

By MicroRNAs (miRNAs)

Here small RNA molecules bind to a complementary portion in the 3'-UTR of the **mRNA** and prevent it from being translated by ribosomes and/or trigger its destruction. Both these activities take place in P bodies.

By Riboswitches

It turns out that the regulation of the level of certain metabolites is controlled by riboswitches. A riboswitch is a part of a molecule of messenger RNA (mRNA) with a specific binding site for the metabolite (or a close relative).

Examples:

- If thiamine pyrophosphate (the active form of thiamine [vitamin B_1]) is available in the culture medium of *E. coli*,
  - It binds to a messenger RNA whose protein product is an enzyme needed to synthesize thiamine from the ingredients in minimal medium.
  - Binding induces an allosteric shift in the structure of the mRNA so that it can no longer bind to a ribosome and thus cannot be translated into the enzyme.
  - E. coli no longer wastes resources on synthesizing a vitamin that is available preformed.

  A thiamine pyrophosphate riboswitch has also been found in plants, archaea, and Neurospora. The one in Neurospora regulates genes involved in vitamin B_1 metabolism by alternative splicing of their transcripts. (Other riboswitches act on transcription rather than translation
- If vitamin B_{12} is present in the cell, it binds to the mRNA which encodes a protein needed to import the vitamin from the culture medium. This, too, induces an allosteric shift in the mRNA that prevents it from binding a ribosome. *E. coli* no longer

wastes resources on synthesizing a transporter for a vitamin that it already has enough of.
- Some Gram-positive bacteria (*E. coli* is Gram-negative) control the level of a sugar needed to synthesize their cell wall with a riboswitch. In this case, as the concentration of the sugar builds up, it binds to the messenger RNA (mRNA) whose product is the enzyme that makes the sugar. This causes the mRNA to self-destruct so production of the enzyme — and thus the sugar — ceases.

It has been suggested that these regulatory mechanisms, which do not involve any protein, are a relict from an "RNA world".

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6.5: RNA Editing

Occasionally researches encounter a gene with a sequence of nucleotides that does **not match exactly** that in its RNA product:

- messenger RNA (mRNA)
- ribosomal RNA (rRNA)
- transfer RNA (tRNA)
- microRNA (miRNA)

If the product is **mRNA**, some of the codons in the open reading frame (ORF) of the gene specify different amino acids from those in the protein translated from the mRNA of the gene.

The reason is **RNA editing**: the alteration of the sequence of nucleotides in the RNA

- after it has been transcribed from DNA but
- before it is translated into protein

RNA editing occurs by two distinct mechanisms:

- **Substitution Editing**: chemical alteration of individual nucleotides (the equivalent of point mutations).

  These alterations are catalyzed by **enzymes** that recognize a specific target sequence of nucleotides (much like restriction enzymes):

  - cytidine deaminases that convert a C in the RNA to uracil (U);
  - adenosine deaminases that convert an A to inosine (I), which the ribosome translates as a G. Thus a CAG codon (for Gln) can be converted to a CGG codon (for Arg).

- **Insertion/Deletion Editing**: insertion or deletion of nucleotides in the RNA.

  These alterations are mediated by **guide RNA** molecules that

  - base-pair as best they can with the RNA to be edited and
  - serve as a **template** for the addition (or removal) of nucleotides in the target

Substitution Editing

The human *APOB* gene

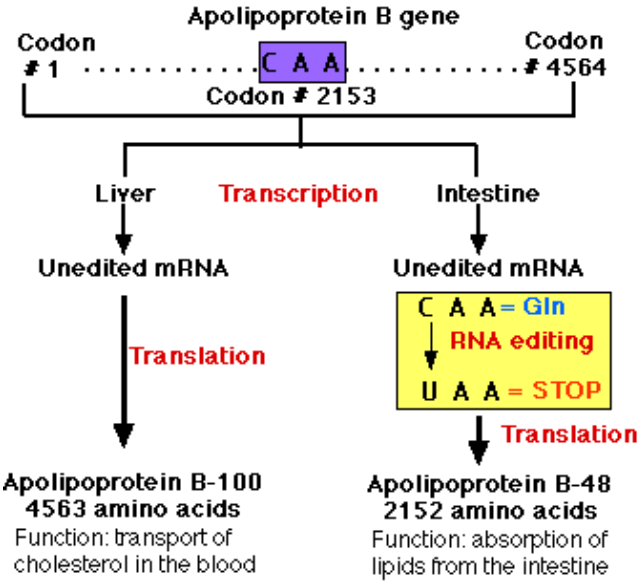


Figure 6.5.1 The Human APOB Gene

Humans have a single locus encoding the **APOB** gene.

- It contains 29 exons (separated by 28 introns).
- The exons contain a total of 4564 codons.
- Codon 2153 is CAA, which is a codon for the amino acid glutamine (Gln).
- The gene is expressed in cells of both the liver and the intestine.
- In both locations, transcription produces a **pre-messenger RNA** that must be spliced to produce the mRNA to be translated into protein.
- In the **Liver**. Here the process occurs normally producing **apolipoprotein B-100** — a protein containing 4,563 amino acids — that is essential for the transport of cholesterol and other lipids in the blood.
- In the **Intestine**
  - In the cells of the intestine, an additional step of pre-mRNA processing occurs: the chemical modification of the C nucleotide in Codon 2153 (CAA) into a U.
  - This **RNA editing** changes the codon from one encoding the amino acid glutamine (Gln) to a STOP codon (UAA)
  - The modification is catalyzed by the enzyme **cytidine deaminase** that
    - recognizes the sequence of the RNA at that one place in the molecule and
    - catalyzes the deamination of C thus forming U.
  - Translation of the mRNA stops at codon #2153 forming **apolipoprotein B-48** — a protein containing 2152 amino acids — that aids in the absorption of dietary lipids from the contents of the intestine.

DNA can also be edited. B cells express another **cytidine deaminase** (called **a**ctivation-**i**nduced **d**eaminase or **AID**) that is essential for both class switch recombination (**CSR**) and somatic hypermutation (**SHM**) of antibody genes. Humans with disabling mutations in the gene for this enzyme produce only IgM antibodies. However, here the enzyme is acting on DNA, not RNA. In attempting to repair the mismatch formed (dC•dG converted to dU•dG), the normal DNA repair machinery of the cell produces CSR or SHM as the situation warrants. (This process is also responsible for the occasional aberrant translocation of the heavy-chain gene segments to a proto-oncogene. The result is a B-cell cancer — a lymphoma or leukemia.)

Other examples of substitution editing

- Some mRNAs, tRNAs, and rRNAs in both the mitochondria and chloroplasts of **plants**;
- mRNAs encoding subunits of some receptors of neurotransmitters in the mammalian brain, e.g.,
  - the AMPA receptor for Glu

- a serotonin receptor
- a tRNA in the mitochondria of the duckbill platypus

Insertion/Deletion Editing

The gene in mitochondria of Trypanosoma brucei

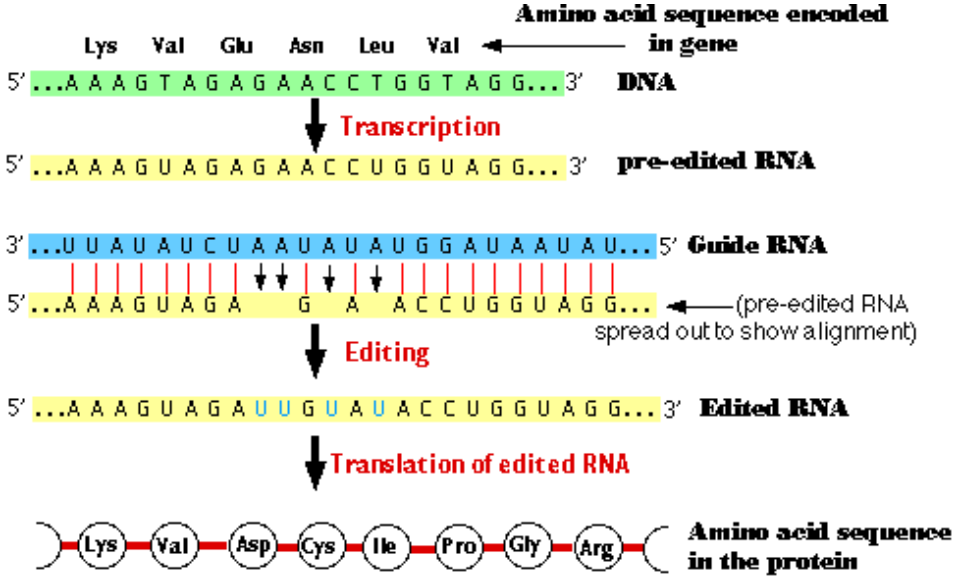


Figure 6.5.2 Insertion/ Deleting Editing

Several genes encoded in the mitochondrial DNA of this species (the cause of sleeping sickness in humans) encode transcripts that must be edited to make the mRNA molecules that will be translated into protein.

Editing requires a special class of RNA molecules called **guide RNA** (**gRNA**).

These small molecules have sequences that are complementary to the region around the site to be edited. The guide RNA base-pairs — as best it can — with this region. Note that in addition to the usual purine-pyrimidine pairing of **C-G** and **A-U**, **G-U** base-pairing can also occur.

Because of the lack of precise sequence complementarity, bulges occur either

- in the guide RNA where, usually, there are **A**s not found in the transcript to be edited (as shown here) or
- in the transcript to be edited.

The bulges are eliminated by cutting the backbone of the shorter molecule and inserting complementary bases.

- In the first case (shown here) this produces **insertions** (here of **U**s)
- In the second case (not shown) this produces **deletions**.

Note that in the example shown here, the insertion of 4 nucleotides has created a frameshift so that the amino acids encoded downstream (after **Val**) in the edited RNA are entirely different from those specified by the gene itself.

Other examples of insertion/deletion editing

Insertion/deletion editing has also been found occur with

- mRNA, rRNA, and tRNA transcripts in the mitochondria of the slime mold **Physarum polycephalum**
- in measles virus transcripts

Why RNA Editing?

Good question. Some possibilities:

- So RNA editing appears to be here to stay. In fact, defects in RNA editing are associated with some human cancers as well as with amyotrophic lateral sclerosis (ALS — "Lou Gehrig's disease").

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6.6: Expressed Sequence Tags

Only a very small percentage (1.2% in humans) of the DNA in vertebrate genomes encodes proteins (the "proteome") because the exons of most genes are separated by much-longer introns between our genes lie vast amounts of DNA much of which appears to regulate the expression of our genes but is not transcribed and translated into a protein product. So even when the complete sequence of a genome is known, it is often difficult to spot particular genes (open reading frames or ORFs).

One approach to solving the problem is to examine a **transcriptome** of the organism. Most commonly this is defined as: All the messenger RNA (mRNA) molecules transcribed from the genome. It is "a" transcriptome, not "the" transcriptome, because what genes are transcribed in a cell depends on the kind of cell (e.g., liver cell vs. lymphocyte) and what the cell is doing at that time, e.g.,

- getting ready to divide by mitosis;
- responding to the arrival of a hormone or cytokine;
- getting ready to secrete a protein product.

Expressed Sequence Tags (ESTs)

ESTs are short (200–500 nucleotides) DNA sequences that can be used to identify a gene that is being expressed in a cell at a particular time.

The Procedure:

- Isolate the messenger RNA (mRNA) from a particular tissue (e.g., liver)
- Treat it with reverse transcriptase. Reverse transcriptase is a DNA polymerase that uses RNA as its template. Thus it is able to make genetic information flow in the reverse (RNA ->DNA) of its normal direction (DNA -> RNA).
- This produces complementary DNA (cDNA). Note that cDNA differs from the normal gene in lacking the intron sequences.
- Sequence 200–500 nucleotides at both the 5′ and 3′ ends of each cDNA.
- Examine the database of the organism's genome to find a matching sequence.

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6.7: Ribosomal RNA (rRNA) Gene Cluster

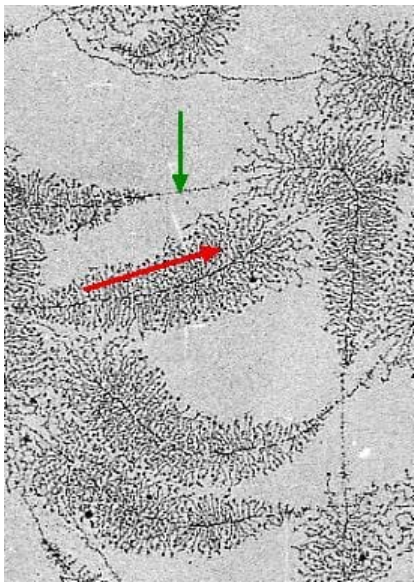


Figure 6.7.1 Transcription of DNA encoding

The above picture shows an electron micrograph (26,500x) showing transcription of the DNA encoding **ribosomal RNA** (rRNA) molecules in the nucleolus of a developing egg cell of the spotted newt.

Eukaryotes have several hundred identical genes encoding ribosomal RNA.

The long filaments (green arrow) are DNA molecules coated with proteins. The fibers extending in clusters from the main axes are molecules of ribosomal RNA which will be used in the construction of the cell's ribosomes.

Note how transcription begins at one end of each gene, with the RNA molecules getting longer (red arrow) as they proceed toward completion.

Note also the large number (up to 100) of RNA molecules that are transcribed **simultaneously** from each gene.

The portions of DNA bare of RNA appear to be genetically inactive. (Courtesy of O. L. Miller, Jr., and Barbara R. Beatty, Biology Division, Oak Ridge National Laboratory.)

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UNIT 7: CELL DIVISION

7.1: Chromosomes
7.2: The Cell Cycle
7.3: Mitosis
7.4: Polyploidy
7.5: Endoreplication
7.6: Sex Chromosomes
7.7: Meiosis

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7.1: Chromosomes

In **eukaryotes**, chromosomes consist of a **single molecule of DNA** associated with many copies of 5 kinds of **histones**. Histones are proteins rich in lysine and arginine residues and thus positively-charged. For this reason they bind tightly to the negatively-charged phosphates in DNA. Cchromosomes have a small number of copies of many different kinds of non-histone proteins. Most of these are **transcription factors** that regulate which parts of the DNA will be transcribed into RNA.

Structure

For most of the life of the cell, chromosomes are too elongated and tenuous to be seen under a microscope. However, before a cell is ready to divide by mitosis, each chromosome is duplicated (during **S phase** of the cell cycle). As mitosis begins, the duplicated chromosomes condense into short (~ 5 µm) structures which can be stained and easily observed under the light microscope. These duplicated chromosomes are called **dyads**.

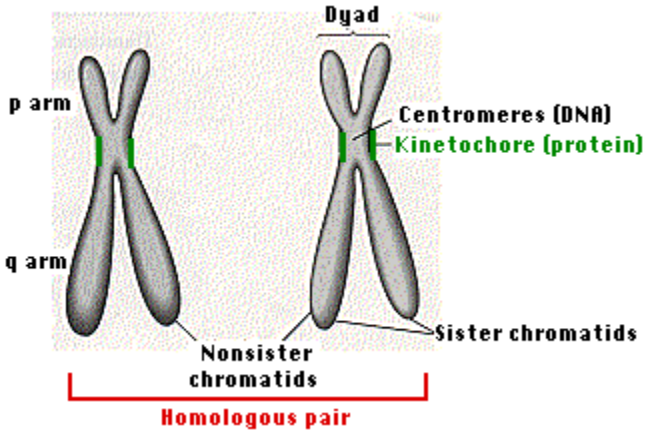


Figure 7.1.1 Dyads

When first seen, the duplicates are held together at their **centromeres**. In humans, the centromere contains 1–10 million base pairs of DNA. Most of this is repetitive DNA: short sequences (e.g., 171 bp) repeated over and over in tandem arrays. While they are still attached, it is common to call the duplicated chromosomes **sister chromatids**, but this should not obscure the fact that each is a bona fide chromosome with a full complement of genes.

The **kinetochore** is a complex of >80 different proteins that forms at each centromere and serves as the attachment point for the spindle fibers that will separate the sister chromatids as mitosis proceeds into anaphase. The shorter of the two arms extending from the centromere is called the **p arm**; the longer is the **q arm**. Staining with the trypsin-giemsa method reveals a series of alternating light and dark bands called **G bands**. G bands are numbered and provide "addresses" for the assignment of gene loci.

Chromosome Numbers

All animals have a characteristic number of chromosomes in their body cells called the **diploid** (or **2n**) number. These occur as **homologous pairs**, one member of each pair having been acquired from the gamete of one of the two parents of the individual whose cells are being examined. The gametes contain the **haploid** number (**n**) of chromosomes. In plants, the haploid stage takes up a larger part of its life cycle.

Table 7.1.1: Diploid numbers of some commonly studied organisms

Homo sapiens (human)	46
Mus musculus (house mouse)	40
Drosophila melanogaster (fruit fly)	8
Caenorhabditis elegans (microscopic roundworm)	12

Saccharomyces cerevisiae (budding yeast)	32
Arabidopsis thaliana (plant in the mustard family)	10
Xenopus laevis (South African clawed frog)	36
Canis familiaris (domestic dog)	78
Gallus gallus (chicken)	78
Zea mays (corn or maize)	20
Muntiacus reevesi (the Chinese muntjac, a deer)	23
Muntiacus muntjac (its Indian cousin)	6
Myrmecia pilosula (an ant)	2
Parascaris equorum var. univalens (parasitic roundworm)	2
Cambarus clarkii (a crayfish)	200
Equisetum arvense (field horsetail, a plant)	216

Karyotypes

The complete set of chromosomes in the cells of an organism is its karyotype. It is most often studied when the cell is at metaphase of mitosis when all the chromosomes are present as dyads. The karyotype of the human female contains 23 pairs of homologous chromosomes: 22 pairs of autosomes and an additional 1 pair of X chromosomes. In contrast, the karyotype of the human male contains the same 22 pairs of autosomes with one X chromosome and one Y chromosome. A gene on the Y chromosome designated SRY is the master switch for making a male. Both X and Y chromosomes are called the sex chromosomes.

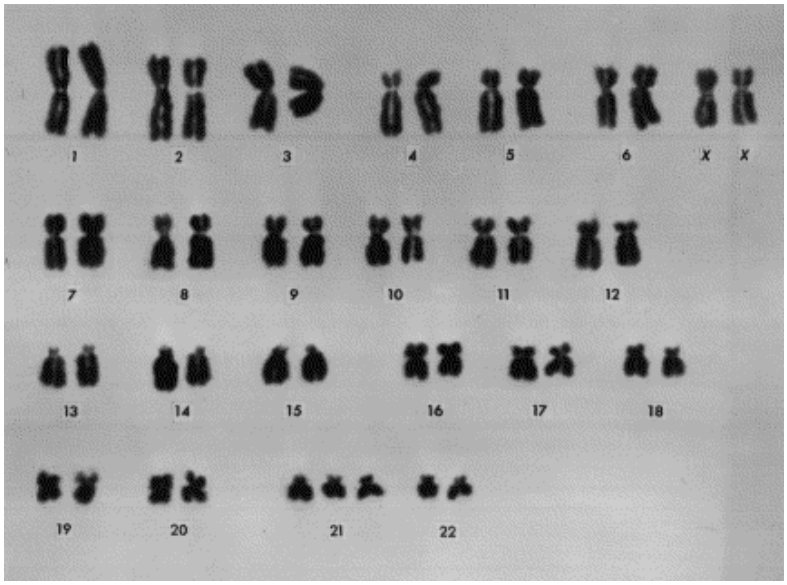


Figure 7.1.2: Human Karyotype

Above is a human karyotype (of which sex?). It differs from a normal human karyotype in having an extra #21 dyad. As a result, this individual suffered from a developmental disorder called **Down Syndrome**. The inheritance of an extra chromosome, is called **trisomy**, in this case trisomy 21. It is an example of **aneuploidy**

Translocations

Karyotype analysis can also reveal translocations between chromosomes. A number of these are associated with cancers, for

example

- the Philadelphia chromosome (Ph^1) formed by a translocation between chromosomes 9 and 22 and a cause of Chronic Myelogenous Leukemia (CML)
- a translocation between chromosomes 8 and 14 that causes Burkitt's lymphoma
- a translocation between chromosomes 18 and 14 that causes B-cell leukemia

Fluorescence in situ Hybridization (FISH)

Figure 7.1.3 provides dramatic evidence of the truth of the story of chromosomes. A piece of single-stranded DNA was prepared that was complementary to the DNA of the human gene encoding the enzyme muscle glycogen phosphorylase. A fluorescent molecule was attached to this DNA. The dyads in a human cell were treated to denature their DNA; that is, to make the DNA single-stranded. When this preparation was treated with the fluorescent DNA, the complementary sequences found and bound each other. This produced a fluorescent spot close to the centromere of each sister chromatid of two homologous dyads (of chromosome 11, upper right). This analytical procedure, which here revealed the **gene locus** for the muscle glycogen phosphorylase gene, is called **fluorescence in situ hybridization** or **FISH**.

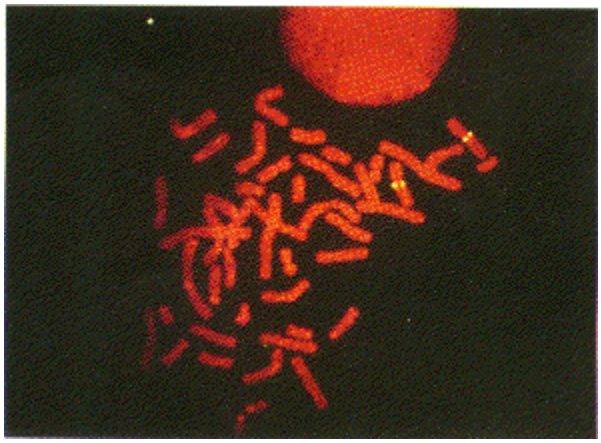


Figure 7.1.3: Location of the gene for muscle glycogen phosphorylase on human chromosome 11 courtesy of David C. Ward

DNA Content

The molecule of DNA in a single human chromosome ranges in size from 50×10^6 nucleotide pairs in the smallest chromosome (stretched full-length this molecule would extend 1.7 cm) up to 250×10^6 nucleotide pairs in the largest (which would extend 8.5 cm). Stretched end-to-end, the DNA in a single human diploid cell would extend over 2 meters. In the intact chromosome, however, this molecule is packed into a much more compact structure. The packing reaches its extreme during mitosis when a typical chromosome is condensed into a structure about 5 µm long (a 10,000-fold reduction in length).

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7.2: The Cell Cycle

A eukaryotic cell cannot divide into two, the two into four, etc. unless two processes alternate:

- **doubling** of its genome (**DNA**) in **S phase** (synthesis phase) of the cell cycle;
- **halving** of that genome during mitosis (**M phase**).

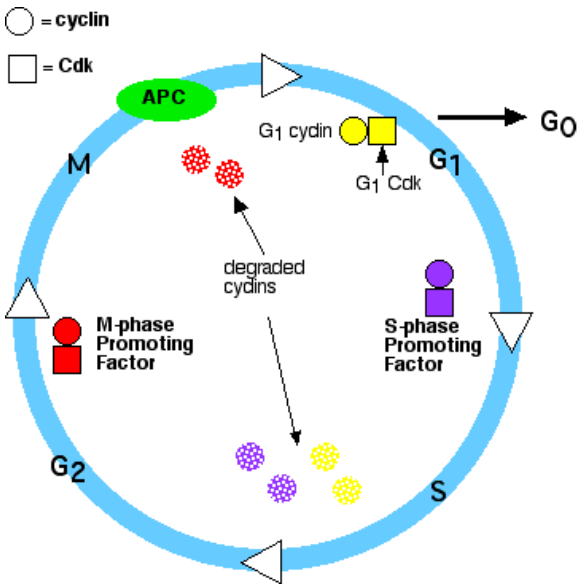


Figure 7.2.1 Cell Cycle: *The cell cycle consists of:* ***G1*** *= growth and preparation of the chromosomes for replication,* **S** *= synthesis of DNA and duplication of the centrosome,* ***G2*** *= preparation for* ***M*** *= mitosis. When a cell is in any phase of the cell cycle other than mitosis, it is often said to be in* ***interphase****.*

Control of the Cell Cycle

The passage of a cell through the cell cycle is controlled by proteins in the cytoplasm. Among the main players in animal cells are:

- Their levels in the cell rise and fall with the stages of the cell cycle.
- Their levels in the cell remain fairly stable, but each must bind the appropriate cyclin (whose levels fluctuate) in order to be activated. They add phosphate groups to a variety of protein substrates that control processes in the cell cycle.
- The **anaphase-promoting complex (APC)**. (The APC is also called the **cyclosome**, and the complex is often designated as the **APC/C**.) The APC/C
  - triggers the events leading to destruction of **cohesin** (as described below) thus allowing the sister chromatids to separate
  - degrades the mitotic (B) cyclins

Steps in the cycle

- A rising level of **G1-cyclins** bind to their Cdks and signal the cell to prepare the chromosomes for replication.
- A rising level of **S-phase promoting factor** (**SPF**) — which includes A cyclins bound to Cdk2 — enters the nucleus and prepares the cell to duplicate its DNA (and its centrosomes).
- As DNA replication continues, cyclin E is destroyed, and the level of mitotic cyclins begins to rise (in G2).
- Translocation of **M-phase promoting factor** (the complex of mitotic [B] cyclins with the M-phase Cdk [Cdk1]) into the nucleus initiates
  - assembly of the mitotic spindle
  - breakdown of the nuclear envelope

    - cessation of all gene transcription
    - condensation of the chromosomes
- These events take the cell to **metaphase** of mitosis.
- At this point, the M-phase promoting factor activates the **anaphase-promoting complex** (**APC/C**) which
    - allows the sister chromatids at the metaphase plate to separate and move to the poles (= anaphase), completing mitosis.

Separation of the sister chromatids depends on the breakdown of the **cohesin** that has been holding them together. It works like this.

- Cohesin breakdown is caused by a protease called **separase** (also known as separin).
- Separase is kept **inactive** until late metaphase by an inhibitory chaperone called **securin**.
- Anaphase begins when the anaphase promoting complex (APC/C) destroys securin (by tagging it with ubiquitin for deposit in a proteasome) thus ending its inhibition of separase and allowing
- separase to break down cohesin

    - destroys **B cyclins**. This is also done by attaching them to **ubiquitin** which targets them for destruction by **proteasomes**.
    - turns on synthesis of G_1 cyclins (D) for the next turn of the cycle.
    - degrades geminin, a protein that has kept the freshly-synthesized DNA in S phase from being re-replicated before mitosis.

This is only one of the mechanisms by which the cell ensures that every portion of its genome is copied once — and only once — during S phase

Some cells deliberately cut the cell cycle short allowing repeated S phases without completing mitosis and/or cytokinesis. This is called **endoreplication**.

Meiosis and the Cell Cycle

The special behavior of the chromosomes in meiosis I requires some special controls. Nonetheless, passage through the cell cycle in meiosis I (as well as meiosis II, which is essentially a mitotic division) uses many of the same players, e.g., **MPF** and **APC**. (In fact, MPF is also called **m**aturation-**p**romoting **f**actor for its role in meiosis I and II of developing oocytes.

Quality Control of the Cell Cycle

The cell has several systems for interrupting the cell cycle if something goes wrong.

DNA damage checkpoints. These sense DNA damage both before the cell enters S phase (a G_1 checkpoint) as well as after S phase (a G_2 checkpoint). Damage to DNA before the cell enters S phase inhibits the action of Cdk2 thus stopping the progression of the cell cycle until the damage can be repaired. If the damage is so severe that it cannot be repaired, the cell self-destructs by apoptosis. Damage to DNA after S phase (the G_2 checkpoint), inhibits the action of Cdk1 thus preventing the cell from proceeding from G_2 to mitosis. A check on the successful replication of DNA during S phase. If replication stops at any point on the DNA, progress through the cell cycle is halted until the problem is solved.

Spindle checkpoints. Some of these that have been discovered to detect any failure of spindle fibers to attach to **kinetochores** and arrest the cell in **metaphase** until all the kinetochores are attached correctly. They detect improper alignment of the spindle itself and block cytokinesis. Furthermore, they trigger apoptosis if the damage is irreparable. All the checkpoints examined require the services of a complex of proteins. Mutations in the genes encoding some of these have been associated with cancer; that is, they are oncogenes. This should not be surprising since checkpoint failures allow the cell to continue dividing despite damage to its integrity.

Examples of checkpoints

p53

The p53 protein senses DNA damage and can halt progression of the cell cycle in G_1 (by blocking the activity of Cdk2). Both copies of the *p53* gene must be mutated for this to fail so mutations in *p53* are recessive, and *p53* qualifies as a **tumor suppressor** gene.

The p53 protein is also a key player in **apoptosis**, forcing "bad" cells to commit suicide. So if the cell has only mutant versions of the protein, it can live on — perhaps developing into a cancer. More than half of all human cancers do, in fact, harbor *p53* mutations and have no functioning p53 protein.

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7.3: Mitosis

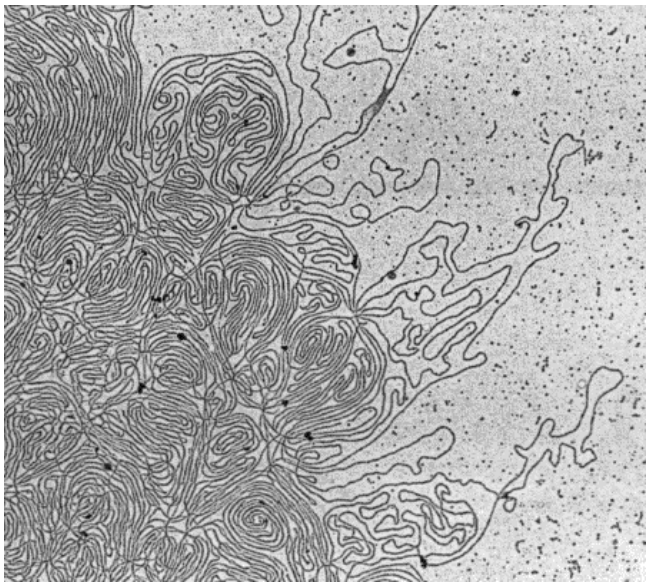


Figure 7.3.1: DNA released from human chromosome courtesy J. R. Paulson and U. C. Laemmli

This image provides a graphic illustration of the problem. It shows a bit (no more than 3%) of the single molecule of DNA released from a single human chromosome. (The chromosome was treated to remove its histones). Remembering that this is 3% of the DNA of only one of the 46 chromosomes in the human diploid cell, you can appreciate the problem faced by the cell of how to separate without error these great lengths of DNA without creating horrible tangles.

The solution to this problem is:

1. Duplicate each chromosome during the S phase of the cell cycle.
2. This produces dyads, each made up of 2 identical sister chromatids. These are held together by a ring of proteins called cohesin.
3. Condense the chromosomes into a compact form. This requires ATP and protein complexes called condensins.
4. Separate the sister chromatids and
5. Distribute these equally between the two daughter cells.

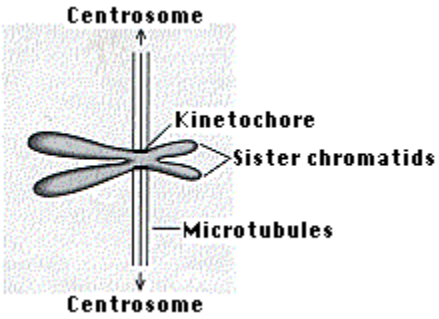


Figure 7.3.2: Kinetochore

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7.4: Polyploidy

Cells (and their owners) are polyploid if they contain **more than two haploid (n) sets** of chromosomes; that is, their chromosome number is some multiple of **n** greater than the **2n** content of diploid cells. For example, triploid (**3n**) and tetraploid cell (**4n**) cells are polyploid.

Polyploidy in plants

Polyploidy is very common in plants, especially in angiosperms. From 30% to 70% of today's angiosperms are thought to be polyploid. Species of coffee plant with 22, 44, 66, and 88 chromosomes are known. This suggests that the ancestral condition was a plant with a haploid (**n**) number of 11 and a diploid (**2n**) number of 22, from which evolved the different polyploid descendants. In fact, the chromosome content of most plant groups suggests that the basic angiosperm genome consists of the genes on 7–11 chromosomes. Domestic wheat, with its 42 chromosomes, is probably hexaploid (**6n**), where **n** (the ancestral haploid number) was 7.

Some other examples:

Plant	Probable ancestral haploid number	Chromosome number	Ploidy level
domestic oat	7	42	**6n**
peanut	10	40	**4n**
sugar cane	10	80	**8n**
banana	11	22, 33	**2n**, **3n**
white potato	12	48	**4n**
tobacco	12	48	**4n**
cotton	13	52	**4n**
apple	17	34, 51	**2n**, **3n**

Polyploid plants not only have larger cells but the plants themselves are often larger. This has led to the deliberate creation of polyploid varieties of such plants as watermelons, marigolds, and snapdragons.

Origin of Polyploidy

Polyploidy has occurred often in the evolution of plants. The process can begin if **diploid** (**2n**) gametes are formed. These can arise in at least two ways.

- The gametes may be formed by mitosis instead of meiosis.
- Plants, in contrast to animals, form germ cells (sperm and eggs) from somatic tissues. If the chromosome content of a precursor somatic cell has accidentally doubled (e.g., as a result of passing through S phase of the cell cycle without following up with mitosis and cytokinesis), then gametes containing **2n** chromosomes are formed.

Polyploidy also occurs naturally in certain plant **tissues**.

- As the endosperm (**3n**) develops in corn (maize) kernels (*Zea mays*), its cells undergo successive rounds (as many as 5) of endoreplication producing nuclei that range as high as **96n**.
- When rhizobia infect the roots of their legume host, they induce the infected cells to undergo endoreplication producing cells that can become **128n** (from 6 rounds of endoreplication).

Polyploidy can also be induced in the plant-breeding laboratory by treating dividing cells with colchicine. This drug disrupts microtubules and thus prevents the formation of a spindle. Consequently, the duplicated chromosomes fail to separate in mitosis.

Onion cells exposed to colchicine for several days may have over 1000 chromosomes inside.

Polyploidy and Speciation

When a newly-arisen tetraploid (**4n**) plant tries to breed with its ancestral species (a **backcross**), triploid offspring are formed. These are sterile because they cannot form gametes with a balanced assortment of chromosomes. However, the tetraploid plants can breed with each other. So in one generation, a new species has been formed. Polyploidy even allows the formation of new species derived from different ancestors.

In 1928, the Russian plant geneticist Karpechenko produced a new species by crossing a cabbage with a radish. Although belonging to different genera (**Brassica** and **Raphanus** respectively), both parents have a diploid number of 18. Fusion of their respective gametes (**n**=9) produced mostly infertile hybrids. However, a few fertile plants were formed, probably by the spontaneous doubling of the chromosome number in somatic cells that went on to form gametes (by meiosis). Thus these contained 18 chromosomes — a complete set of both cabbage (**n**=9) and radish (**n**=9) chromosomes. Fusion of these gametes produced vigorous, fully-fertile, polyploid plants with 36 chromosomes. (They had the roots of the cabbage and the leaves of the radish.)

These plants could breed with each other, but not with either the cabbage or radish ancestors, so Karpechenko had produced a new species. The process also occurs in nature. Three species in the mustard family (Brassicaceae) appear to have arisen by hybridization and polyploidy from three other ancestral species:

- ***B. oleracea*** (cabbage, broccoli, etc.) hybridized with ***B. nigra*** (black mustard) ⟶ ***B. carinata*** (Abyssinian mustard).
- ***B. oleracea*** x ***B. rapa*** (turnips) ⟶ ***B. napus*** (rutabaga)
- ***B. nigra*** x ***B. rapa*** ⟶ ***B. juncea*** (leaf mustard)

Modern wheat and perhaps some of the other plants listed in the table above have probably evolved in a similar way.

Polyploidy in Animals

Polyploidy is much rarer in animals. It is found in some insects, fishes, amphibians, and reptiles. Until recently, no polyploid **mammal** was known. However, the 23 September 1999 issue of **Nature** reported that a polyploid (tetraploid; **4n** = 102) rat has been found in Argentina. Polyploid cells are larger than diploid ones; not surprising in view of the increased amount of DNA in their nucleus. The liver cells of the Argentinian rat are larger than those of its diploid relatives, and its sperm are huge in comparison. Normal mammalian sperm heads contain some 3.3 picograms (10^{-12} g) of DNA; the sperm of the rat contains 9.2 pg. Although only one mammal is known to have all its cells polyploid, many mammals have polyploid cells in certain of their organs, e.g, the liver.

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7.5: Endoreplication

Endoreplication is the replication of DNA during the S phase of the cell cycle without the subsequent completion of mitosis and/or cytokinesis. Endoreplication is also known as endoreduplication. Endoreplication occurs in certain types of cells in both animals and plants. There are several variations:

- replication of DNA with completion of mitosis, but no cytokinesis.
- repeated replication of DNA without forming new nuclei in telophase. This can result in:
  1. Polyploidy: the replicated chromosomes retain their individual identity.
  2. Polyteny: the replicated chromosomes remain in precise alignment forming "giant" chromosomes.
  3. various intermediate conditions between 1 and 2

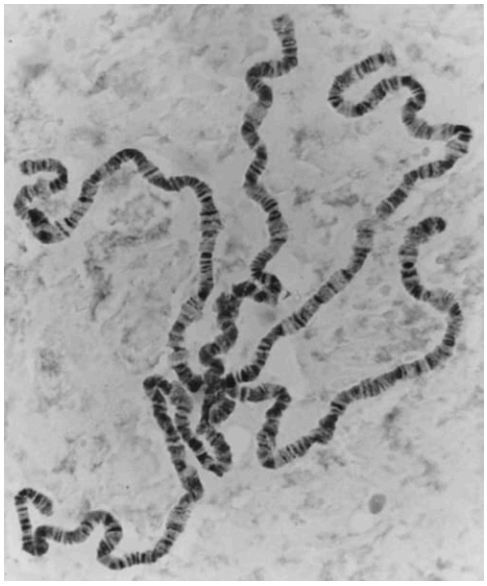


Figure 7.5.1 Polytene Chromosomes courtesy of B. P. Kaufmann

The photomicrograph shows the polytene chromosomes in a salivary gland cell of a *Drosophila melanogaster* larva. Such chromosomes are found in other large, active cells as well.

- Each of Drosophila's 4 pairs of chromosomes has undergone 10 rounds of DNA replication.
- The maternal and paternal homologs — as well as all their duplicates — are aligned in exact register with each other.
- So each chromosome consists of a cable containing 2048 identical strands of DNA.
- These are so large that they can be seen during interphase; even with a low-power light microscope.

Function of polyteny

The probable answer: gene amplification. Having multiple copies of genes permits a high level of gene expression; that is, abundant transcription and translation to produce the gene products. This would account for polyteny being associated with large, metabolically active cells (like salivary glands). Polytene chromosomes are subdivided into some 5,000 dense bands separated by light interbands. The bands are further subdivided into:

- dark bands of heterochromatin where the DNA is tightly compacted and there is little gene transcription;
- gray bands of euchromatin where the DNA is more loosely compacted and there is active gene transcription.

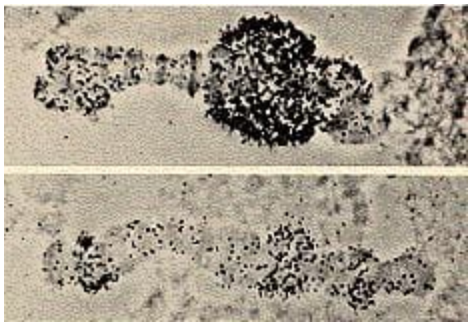


Figure 7.5.3 Changes in puffing patterns of Chromosomes courtesy of Dr. Michael Ashburner, University of Cambridge

These eight photomicrographs () show the changes in the puffing pattern of equivalent segments of chromosome 3 in *Drosophila melanogaster* over the course of some 20 hours of normal development.

Note that during this period, when the larvae were preparing to pupate, certain puffs formed, regressed, and formed again. However, the order in which they did often differed. For example, in the larva, band 62E becomes active before 63E (c, d, and e), but when pupation begins, the reverse is true (g, h).

In general, early puffs reflect the activation of genes encoding transcription factors. These proteins then bind to the promoters of other genes, turning them on and causing a puff to appear at their loci.

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7.6: Sex Chromosomes

The nuclei of human cells contain 22 autosomes and 2 sex chromosomes. In females, the sex chromosomes are the 2 **X chromosomes**. Males have one X chromosome and one **Y chromosome**. The presence of the Y chromosome is decisive for unleashing the developmental program that leads to a baby boy.

The Y Chromosome

In making sperm by meiosis, the X and Y chromosomes must separate in anaphase just as homologous autosomes do. This occurs without a problem because, like homologous autosomes, the X and Y chromosome synapse during prophase of meiosis I. There is a small region of homology shared by the X and Y chromosome and synapsis occurs at that region.

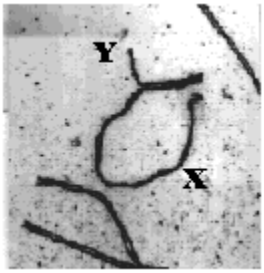


Figure 7.6.1 Synapsis of the X and Y chromosomes courtesy of C. Tease

This image, shows synapsis of the X and Y chromosomes of a mouse during prophase of meiosis I. Crossing over occurs in two regions of pairing, called the **pseudoautosomal regions**. These are located at opposite ends of the chromosome.

The Pseudoautosomal Regions

The **pseudoautosomal regions** get their name because any genes located within them (so far only 9 have been found) are inherited just like any autosomal genes. Males have two copies of these genes: one in the pseudoautosomal region of their Y, the other in the corresponding portion of their X chromosome. So males can inherit an allele originally present on the X chromosome of their father and females can inherit an allele originally present on the Y chromosome of their father.

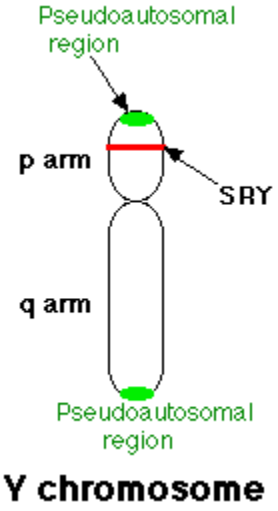


Figure 7.6.2 Human Y Chromosome

Genes outside the pseudoautosomal regions

Although 95% of the Y chromosome lies between the pseudoautosomal regions, only 27 different functional genes have been

found here. Over half of this region is genetically-barren heterochromatin. Of the 27 genes found in the euchromatin, some encode proteins used by all cells. The others encode proteins that appear to function only in the testes. A key player in this latter group is ***SRY***.

SRY

SRY (for **s**ex-determining **r**egion **Y**) is a gene located on the short (p) arm just outside the pseudoautosomal region. It is the master switch that triggers the events that converts the embryo into a male. Without this gene, you get a female instead.

What is the evidence?

1. On very rare occasions aneuploid humans are born with such karyotypes as XXY, XXXY, and even XXXXY. Despite their extra X chromosomes, all these cases are male.
2. This image shows two mice with an **XX karyotype** (and thus they should be female). However, as you may be able to see, they have a male phenotype. This is because they are transgenic for ***SRY***. Fertilized XX eggs were injected with DNA carrying the *SRY* gene.

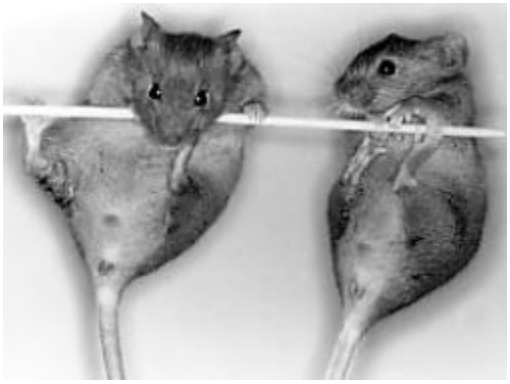


Figure 7.6.3 Transgenic mice courtesy of Robin Lovell-Badge from Nature 351:117, 1991

Although these mice have testes, male sex hormones, and normal mating behavior, they are sterile.

3. Another rarity: XX humans with testicular tissue because a translocation has placed the *SRY* gene on one of the X chromosomes
4. Still another rarity that demonstrates the case: women with an XY karyotype who, despite their Y chromosome, are female because of a destructive mutation in *SRY*.

In 1996, a test based on a molecular probe for *SRY* was used to ensure that potential competitors for the women's Olympic events in Atlanta had no *SRY* gene. But because of possibilities like that in case 4, this testing is no longer used to screen female Olympic athletes.

The X Chromosome

The X chromosome carries nearly 1,000 genes but few, if any, of these have anything to do directly with sex. However, the inheritance of these genes follows special rules. These arise because:

- males have only a single X chromosome
- almost all the genes on the X have no counterpart on the Y; thus
- any gene on the X, **even if recessive in females**, will be expressed in males.

Genes inherited in this fashion are described as sex-linked or, more precisely, **X-linked**.

X-Linkage example

Hemophilia is a blood clotting disorder caused by a mutant gene encoding either

- clotting **factor VIII**, causing hemophilia A or
- clotting **factor IX**, causing hemophilia B.

Both genes are located on the X chromosome (shown here in red). With only a single X chromosome, males who inherit the defective gene (always from their mother) will be unable to produce the clotting factor and suffer from difficult-to-control episodes of bleeding. In heterozygous females, the unmutated copy of the gene will provide all the clotting factor they need. Heterozygous females are called **"carriers"** because although they show no symptoms, they pass the gene on to approximately half their sons, who develop the disease, and half their daughters, who also become carriers.

	X	Y
X	XX	XY
x^h	x^hX	x^hY

Women rarely suffer from hemophilia because to do so they would have to inherit a defective gene from their father as well as their mother. Until recently, few hemophiliacs ever became fathers.

X-chromosome Inactivation (XCI)

Human females inherit two copies of every gene on the X chromosome, whereas males inherit only one (with some exceptions: the 9 pseudoautosomal genes and the small number of "housekeeping" genes found on the Y). But for the hundreds of other genes on the X, are males at a disadvantage in the amount of gene product their cells produce? The answer is no, because females have only a single **active** X chromosome in each cell.

During interphase, chromosomes are too tenuous to be stained and seen by light microscopy. However, a dense, stainable structure, called a **Barr body** (after its discoverer) is seen in the interphase nuclei of female mammals. The Barr body is one of the X chromosomes. Its compact appearance reflects its inactivity. So, the cells of females have only one functioning copy of each X-linked gene — the same as males.

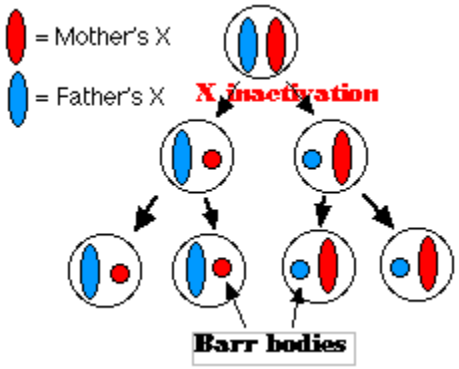


Figure 7.6.4 Genetic Mosaic due to XChromosome Inactivation

X-chromosome inactivation occurs early in embryonic development. In a given cell, which of a female's X chromosomes becomes inactivated and converted into a Barr body is a matter of chance (except in marsupials like the kangaroo, where it is always the father's X chromosome that is inactivated). After inactivation has occurred, all the descendants of that cell will have the same chromosome inactivated. Thus X-chromosome inactivation creates clones with differing effective gene content. An organism whose cells vary in effective gene content and hence in the expression of a trait, is called a **genetic mosaic**.

Mechanism of X-chromosome inactivation

Inactivation of an X chromosome requires a gene on that chromosome called ***XIST***.

- *XIST* is transcribed into a **long noncoding RNA**.
- XIST RNA accumulates along the X chromosome containing the active *XIST* gene and proceeds to inactivate all (or almost all) of the hundreds of other genes on that chromosome.

- **Barr bodies** are inactive X chromosomes "painted" with XIST RNA.

The Sequence of Events in Mice

- During the first cell divisions of the female mouse zygote, the *XIST* locus on the father's X chromosome is expressed so most of his X-linked genes are silent.
- By the time the blastocyst has formed, the silencing of the paternal X chromosome still continues in the trophoblast (which will go on to form the placenta) but
- in the inner cell mass (the ICM, which will go on to form the embryo) transcription of *XIST* **ceases** on the paternal X chromosome allowing its hundreds of other genes to be expressed. The shut-down of the *XIST* locus is done by methylating *XIST* regulatory sequences. So the pluripotent stem cells of the ICM express both X chromosomes.
- However, as embryonic development proceeds, X-chromosome inactivation begins again. But this time it is entirely random. There is no predicting whether it will be the **maternal X** or the **paternal X** that is inactivated in a given cell.

Some genes on the X chromosome escape inactivation

What about those 18 genes that are found on the Y as well as the X? There should be no need for females to inactivate one copy of these to keep in balance with the situation in males. And, as it turns out, these genes escape inactivation in females. Just how they manage this is still under investigation.

X-chromosome Abnormalities

As we saw above, people are sometimes found with abnormal numbers of X chromosomes. Unlike most cases of aneuploidy, which are lethal, the phenotypic effects of aneuploidy of the X chromosome are usually not severe.

Examples:

- Females with but a single intact X chromosome (usually the one she got from her mother) in some (thus a genetic mosaic) or all of her cells show a variable constellation of phenotypic traits called **Turner syndrome**. For those girls that survive to birth, the phenotypic effects are generally mild because each cell has a single functioning X chromosome like those of XX females. Number of Barr bodies = zero.
- **XXX**, **XXXX**, **XXXXX** karyotypes: all females with mild phenotypic effects because in each cell all the extra X chromosomes are inactivated. Number of Barr bodies = number of X chromosomes minus one.
- **Klinefelter's syndrome**: people with XXY or XXXY karyotypes are males (because of their Y chromosome). But again, the phenotypic effects of the extra X chromosomes are mild because, just as in females, the extra Xs are inactivated and converted into Barr bodies.

Sex Determination in Other Animals

Although the male fruit fly, *Drosophila melanogaster*, is X-Y, the Y chromosome does not dictate its maleness but rather the absence of a second X. Furthermore, instead of females shutting down one X to balance the single X of the males — as we do — male flies double the output of their single X relative to that of females.

In birds, moths, schistosomes, and some lizards, the male has two of the same chromosome (designated ZZ), whereas the female has "heterogametic" chromosomes (designated Z and W). In chickens, a single gene on the Z chromosome (designated *DMRT1*), when present in a double dose (ZZ), produces males while the presence of only one copy of the gene produces females (ZW).

Environmental Sex Determination

In some cold-blooded vertebrates such as

- fishes
- reptiles (e.g. certain snakes, lizards, turtles, and all crocodiles and alligators)
- invertebrates (e.g. certain crustaceans),

sex is determined **after fertilization** — not by sex chromosomes deposited in the egg.

The choice is usually determined by the **temperature** at which early embryonic development takes place.

- In some cases (e.g. many turtles and lizards), a higher temperature during incubation favors the production of females.
- In other cases (e.g., alligators), a higher temperature favors the production of males.

Even in cases (e.g. some lizards) where there are sex chromosomes, a high temperature can convert a genotypic male (ZZ) into a female.

Hermaphrodites

Hermaphrodites have both male and female sex organs. Many species of fish are hermaphroditic.

Some start out as one sex and then, in response to stimuli in their environment, switch to the other.

Other species have both testes and ovaries at the same time (but seldom fertilize themselves). However, populations of C. elegans consist mostly of hermaphrodites and these only fertilize themselves.

Hermaphroditic fishes have no sex chromosomes.

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7.7: Meiosis

Mitosis produces two cells with the same number of chromosomes as the parent cell. Mitosis of a **diploid** cell (**2n**) produces two diploid daughter cells. If two diploid cells went on to participate in sexual reproduction, their fusion would produce a tetraploid (**4n**) **zygote**. The solution for this problem is Meiosis.

Meiosis

Meiosis is a process of cell division in **eukaryotes** characterized by:

- two consecutive divisions: **meiosis I** and **meiosis II**
- no DNA synthesis (no S phase) between the two divisions
- the result: 4 cells with half the number of chromosomes of the starting cell, e.g., **2n** → **n**

Fusion of two such cells produces a **2n** zygote.

Meiosis in Animals

Used to produced the **gametes**: sperm and eggs

Meiosis in Plants

Used to produce **spores**. Spores are the start of the **gametophyte generation** which, in time, will produce gametes (by mitosis because the starting cells are already haploid).

Meiosis I

Prophase of meiosis I (**prophase I**) is a more elaborate process than prophase of mitosis (and usually takes much longer).

Here is a brief overview of the process. A more detailed view is provided below.

- When the chromosomes first become visible they are already doubled, each homologue having been duplicated during the preceding S phase.
- Result: pairs of **homologous dyads** each dyad consisting of two sister chromatids held together by a protein complex called **cohesin**.
- **Pairing**: Each pair of homologous dyads align lengthwise with each other.
- Result: a **tetrad**. (These structures are sometimes referred to as **bivalents** because at this stage you cannot distinguish the individual sister chromatids under the microscope.)
- The two homologous dyads are held together by
  - one or more **chiasmata** (sing. = chiasma) which form between two **nonsister** chromatids at points where they have crossed over.
  - the **synaptonemal complex** (SC), a complex assembly of proteins (including cohesin)

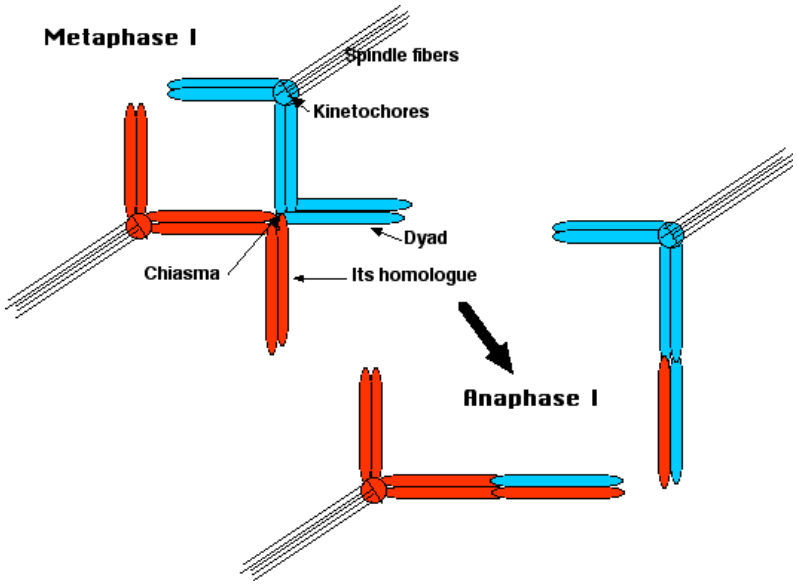


Figure 7.7.1 Meiosis I

At **metaphase I**, microtubules of the spindle fibers attach to the

- sister kinetochores of one homologue, pulling both sister chromatids toward one pole of the cell;
- sister kinetochores of the other homologue pulling those sisters toward the opposite pole.

Result: one homologue is pulled above the metaphase plate, the other below. The chiasmata keep the homologues attached to each other, and the cohesin keeps the sister chromatids together.

At **anaphase I**,

- the cohesin between the chromosome **arms** breaks down allowing
- the chiasmata to slip apart.
- Result: the homologous dyads separate and migrate toward their respective poles.

Meiosis II

Chromosome behavior in meiosis II is like that of mitosis.

- At metaphase II, spindle fibers attach one kinetochore of the dyad to one pole, the other to the opposite pole.
- At anaphase II, the chromatids separate and (each now an independent chromosome) move to their respective poles.

Genetic Recombination

Meiosis not only preserves the genome size of sexually reproducing eukaryotes but also provides three mechanisms to diversify the genomes of the offspring.

Crossing Over

Chiasmata represent points where earlier (and unseen) nonsister chromatids had swapped sections. The process is called crossing over. It is reciprocal; the segments exchanged by each nonsister chromatid are identical (but may carry different alleles).

Each chromatid contains a single molecule of DNA. So the problem of crossing over is really a problem of swapping portions of adjacent DNA molecules. It must be done with great precision so that neither chromatid gains or loses any genes. In fact, crossing over has to be sufficiently precise that not a single nucleotide is lost or added at the crossover point if it occurs within a gene. Otherwise a **frameshift** would result and the resulting gene would produce a defective product or, more likely, no product at all.



Figure 7.7.2 Single Chiasma courtesy of Prof. Bernard John

In the diagram above, only a single chiasma is shown. However, multiple chiasmata are commonly found (in humans the average number of chiasmata per tetrad is just over two). In this photomicrograph, a tetrad of the grasshopper **Chorthippus parallelus** shows 5 chiasmata.

Random Assortment

In meiosis I, the orientation of paternal and maternal homologues at the metaphase plate is random. Therefore, although each cell produced by meiosis contains only one of each homologue, the number of possible combinations of maternal and paternal homologues is 2^n, where **n** = the haploid number of chromosomes. In this diagram, the haploid number is 3, and 8 (2^3) different combinations are produced.

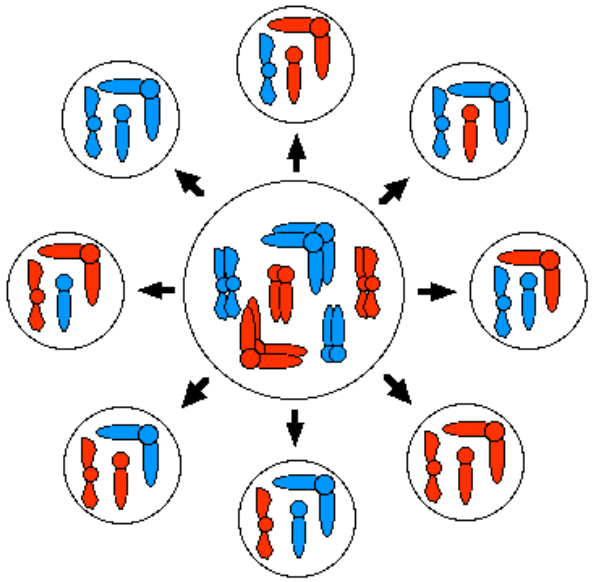


Figure 7.7.3 Random Assortment

Random assortment of homologues in humans produces 2^{23} (8,388,608) different combinations of chromosomes.

Furthermore, because of crossing over, none of these chromosomes is "pure" maternal or paternal. The distribution of recombinant and non-recombinant sister chromatids into the daughter cells at anaphase II is also random.

So I think it is safe to conclude that of all the billions of sperm produced by a man during his lifetime (and the hundreds of eggs that mature over the life of a woman), no two have exactly the same gene content.

Fertilization

By reducing the number of chromosomes from **2n** to **n**,the stage is set for the union of two genomes. If the parents differ genetically, new combinations of genes can occur in their offspring.

Taking these three mechanisms together, I think that it is safe to conclude that no two human beings have ever shared an identical genome **unless** they had an **identical** sibling; that is a sibling produced from the same fertilized egg.

The behavior of chromosomes during meiosis (**2n** → **n**) and fertilization (**n** + **n** → **2n**) provide the structural basis for Mendel's rules of inheritance.

Prophase I — a detailed view

The lengthy and complex events of prophase I can be broken down into 5 stages.

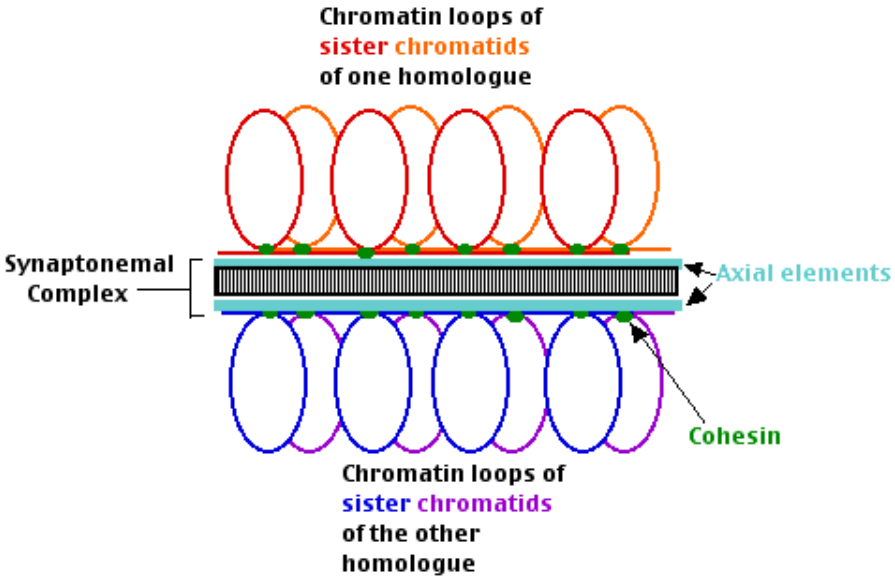


Figure 7.7.4 Prophase I

Leptotene

- All the chromosomes condense.
- Pairing. Homologous dyads (pairs of sister chromatids) find each other and align themselves from end to end with the aid of an **axial element** (that contains cohesin). In budding yeast (and perhaps other eukaryotes) the process follows a period of trial-and-error. Any two dyads pair at their centromeres. If they are not homologs, they separate and try again.
- How the nonsisters recognize their shared regions of DNA homology is uncertain. Double-stranded breaks (DSBs) often occur in the DNA of the chromatids, and these may be necessary for the homologs to recognize each other.

Zygotene

- Synapsis. The **synaptonemal complex** begins to form.
- DNA strands of nonsister chromatids begin the process of **recombination**. How they are able to do so across the synaptonemal complex, which is over 100 nm thick, is unknown.

Pachytene

- Synapsis is now complete.
- **Recombination nodules** appear (at least in some organisms, including humans). They are named for the idea that they represent points where DNA recombination is occurring.
  - There must be at least one for each bivalent if meiosis is to succeed. There are often more, each one presumably representing the point of a crossover.
  - They contain enzymes known to be needed for DNA recombination and repair.
- The steps in recombining DNA continue to the end of pachytene.

Diplotene

- DNA recombination is complete.
- The synaptonemal complex begins to break down.
- The chromatids begin to pull apart revealing
- **chiasmata**. At first the chiasmata are located at the sites of the recombination nodules, but later they migrate towards the ends of the chromatids.

Diakinesis

In some organisms, the chromosomes decondense and begin to be transcribed for a time. This is followed by the chromosomes recondensing in preparation for metaphase I.

In creatures where this does not occur, the chromosomes condense further in preparation for metaphase I.

Quality Control of Meiosis

It shouldn't be surprising that things can go wrong in such a complicated process. However, cells going through meiosis have checkpoints that monitor each pair of homologues for

- proper recombination of their DNA
- correct formation of the synaptonemal complex

Any failure that is detected stops the process and usually causes the cell to self-destruct by apoptosis.

However, despite these checkpoints, errors occasionally do go uncorrected.

Errors in Meiosis

It is estimated that from 10–25% of all human fertilized eggs contain chromosome abnormalities, and these are the most common cause of pregnancy failure (35% of the cases).

These chromosome abnormalities

- arise from errors in meiosis, usually meiosis I;
- occur more often (90%) during egg formation than during sperm formation;
- become more frequent as a woman ages.
- **Aneuploidy** — the gain or loss of whole chromosomes — is the most common chromosome abnormality. It is caused by **nondisjunction**, the failure of chromosomes to correctly separate:
  - homologues during meiosis I or
  - sister chromatids during meiosis II
- Zygotes missing one chromosome ("monosomy") cannot develop to birth (except for females with a single X chromosome).
- Three of the same chromosome ("trisomy") is also lethal except for chromosomes 13, 18, and 21 (trisomy 21 is the cause of Down syndrome).
- Three or more X chromosomes are viable because all but one of them are inactivated.

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UNIT 8: THE GENETIC CONSEQUENCES OF MEIOSIS

8.1: Mendel's Monohybrid Crosses
8.2: Crossing Over and Genetic Recombination in Meiosis
8.3: The Evidence of Creighton and McClintock
8.4: Genetic linkage and Genetic Maps
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8.1: Mendel's Monohybrid Crosses

Gregor Mendel (1822-1884) was an Austrian monk who discovered the basic rules of inheritance. From 1858 to 1866, he bred garden peas in his monastery garden and analyzed the offspring of these matings. The garden pea was good choice of experimental organism because many varieties were available that bred true for clear-cut, **qualitative** traits like

- seed texture (round vs wrinkled)
- seed color (green vs yellow)
- flower color (white vs purple)
- tall vs dwarf growth habit
- and three others that also varied in a qualitative — rather than quantitative — way.

Furthermore, peas are normally **self-pollinated** because the stamens and carpels are enclosed within the petals. By removing the stamens from unripe flowers, Mendel could brush pollen from another variety on the carpels when they ripened.

The First Cross

Mendel crossed a pure-breeding round-seeded variety with a pure-breeding wrinkled-seeded one. The parents (designated the **P** generation) were pure-breeding because each was homozygous for the alleles at the gene locus (on chromosome 7) controlling seed texture (**RR** for round; **rr** for wrinkled).

The results

All the peas produced in the second or **hybrid generation** were round.

Interpretation

All the peas of this **F_1 generation** have an **Rr genotype**. All the haploid sperm and eggs produced by meiosis received one chromosome 7. All the zygotes received one **R** allele (from the round parent) and one **r** allele (from the wrinkled parent). Because the round trait is **dominant**, the **phenotype** of all the seeds was round.

		P gametes (round parent)	
		R	R
P gametes (wrinkled parent)	r	Rr	Rr
	r	Rr	Rr

The Second Cross

Mendel then allowed his hybrid peas to self-pollinate.

The results

The wrinkled trait — which had disappeared in his hybrid generation — reappeared in 25% of the new crop of peas.

Interpretation

Random union of equal numbers of **R** and **r** gametes produced an F_2 generation with 25% **RR** and 50% **Rr** — both with the round phenotype — and 25% **rr** with the wrinkled phenotype.

		F_1 gametes	
		R	r

F_1 gametes	R	RR	Rr
	r	Rr	rr

The Third Cross

Mendel then allowed some of each phenotype in the F_2 generation to self-pollinate. His results:

- All the wrinkled seeds in the F_2 generation produced only wrinkled seeds in the F_3.
- One-third (193/565) of the round F_1 seeds produced only round seeds in the F_3 generation, but
- two-thirds (372/565) of them produced both types of seeds in the F_3 and — once again — in a 3:1 ratio.

Interpretation

One-third of the round seeds and all of the wrinkled seeds in the F_2 generation were homozygous and produced only seeds of the same phenotype. But two thirds of the round seeds in the F_2 were heterozygous and their self-pollination produced both phenotypes in the ratio of a typical F_1 cross.

Phenotype ratios are approximate

The union of sperm and eggs is random. So the pod in the color photo () — with its 9 smooth seeds and 3 wrinkled seeds! — represents something of a statistical fluke. As the size of the sample gets larger, however, chance deviations become minimized and the ratios approach the theoretical predictions more closely. The table shows the actual seed production by ten of Mendel's F_1 plants. While his individual plants deviated widely from the expected 3:1 ratio, the group as a whole approached it quite closely.



Figure 8.1.1 courtesy of Cathie Martin from Cell 12 January 1990

	Round	Wrinkled
1.	45	12
2.	27	8
3.	24	7
4.	19	16
5.	32	11

	Round	Wrinkled
6.	26	6
7.	88	24
8.	22	10
9.	28	6
10.	25	7
Total	**336**	**107**

Mendel's Hypothesis

To explain his results, Mendel formulated a hypothesis that included the following:

1. In the organism there is a pair of factors that controls the appearance of a given characteristic. (We call them **genes**.)
2. The organism inherits these factors from its parents, one from each.
3. Each is transmitted from generation to generation as a discrete, unchanging unit. (The wrinkled seeds in the F_2 generation were no less wrinkled than those in the P generation although they had passed through the round-seeded F_1 generation.)
4. When the gametes are formed, the factors separate and are distributed as units to each gamete. This statement is often called Mendel's **rule of segregation**.
5. If an organism has two unlike factors (we call them alleles) for a characteristic, one may be expressed to the total exclusion of the other (**dominant** vs **recessive**).

The Testcross: A Test of Mendel's Hypothesis

A good hypothesis meets several standards.

- It should provide an adequate explanation of the observed facts.
- If two or more hypotheses meet this standard, the simpler one is preferred.
- It should be able to **predict** new facts.

So if a generalization is valid, then certain specific consequences can be **deduced** from it. To test his hypothesis, Mendel predicted the outcome of a breeding experiment that he had not yet carried out. He crossed heterozygous round peas (**Rr**) with wrinkled (homozygous, **rr**) ones. He predicted that in this case one-half of the seeds produced would be round (**Rr**) and one-half wrinkled (**rr**)

		F1 gametes	
		R	r
P gametes	r	Rr	rr
	r	Rr	rr

To a casual observer in the monastery garden, the cross appeared no different from the **P** cross described above: round-seeded peas being crossed with wrinkled-seeded ones. But Mendel predicted that this time he would produce both round and wrinkled seeds and in a 50:50 ratio. He performed the cross and harvested 106 round peas and 101 wrinkled peas.

This kind of mating is called a **testcross**. It "tests" the genotype in those cases where two different genotypes (like **RR** and **Rr**) produce the same phenotype.

Mendel did not stop here.

- He went on to cross pea varieties that differed in six other qualitative traits. In every case, the results supported his hypothesis.
- He crossed peas that differed in **two traits**. He found that the inheritance of one trait was independent of that of the other

and so framed his second rule: the **rule of independent assortment**.

Mendel's rules today

Little attention was paid when Mendel published his findings in 1866. Not until 1900, 34 years later and 16 years after his death, was his work brought to light. By then, three men — working independently — discovered the same principles. So the present remarkable development of genetics dates from only the start of the 20th century.

The discovery of chromosomes — and their behavior during meiosis (**2n** -> **n**) and fertilization (**n** + **n** -> **2n**) — established the structural basis for Mendel's rules.

What is the status today of Mendel's rules? Although many important exceptions to them have been discovered — three examples:

- both members of many allelic pairs affect the phenotype; that is, neither is fully dominant
- several different pairs of genes — often on different chromosomes — affect a phenotype **additively** with none being fully dominant.
- many gene loci are not inherited independently but show linkage (because they are relatively close together on the same chromosome)

His rules still form the foundation upon which the science of genetics rests.

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8.2: Crossing Over and Genetic Recombination in Meiosis

Crossing over occurs between equivalent portions of two nonsister chromatids.

Each chromatid contains a single molecule of DNA. So the problem of crossing over is really a problem of swapping portions of adjacent DNA molecules.

It must be done with great precision so that neither chromatid gains or loses any genes. In fact, crossing over has to be sufficiently precise that not a single nucleotide is lost or added at the crossover point if it occurs within a gene. Otherwise a **frameshift** would result and the resulting gene would produce a defective product or, more likely, no product at all.

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8.3: The Evidence of Creighton and McClintock

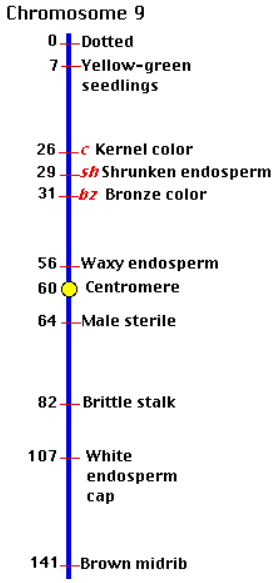


Figure 8.3.1 Unusual Corn Chromosome

Here was an organism with a rare chromosomal aberration that made it possible to distinguish two homologs from each other under the microscope. Furthermore, this unusual chromosome carried the dominant allele for colored kernels (***C***) and the recessive allele for waxy endosperm (***wx***). Its normal-appearing mate carried the recessive allele for colorless kernels (***c***) and the dominant allele for normal (starchy) endosperm (***Wx***). Thus the plant was a dihybrid for these two linked traits and, in addition, one chromosome of the pair was visibly marked at each end.

Creighton and McClintock reasoned that this plant would produce 4 kinds of gametes: The parental kinds (***Cwx*** and ***cWx***) and the recombinant kinds produced by crossing over (***cwx*** and ***CWx***). Fertilization of these gametes by gametes containing a chromosome of normal appearance and both recessive alleles ***cwx*** (a typical testcross) should produce 4 kinds of kernels:

- colored waxy (***Ccwxwx***) kernels
- colorless kernels with normal endosperm (***ccWxwx***)
- colorless waxy (***ccwxwx***) and
- colored kernels with normal endosperm (***CcWxwx***).

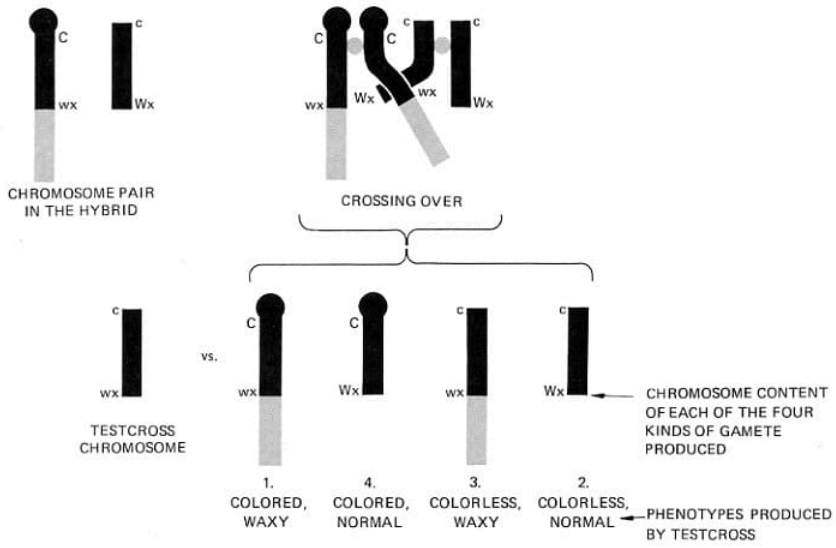


Figure 8.3.2 Creighton - McClintock test cross chromosomes

1. In the first case, there should be one normal chromosome and one extra-long chromosome with the knob at the end.
2. In the second case, both chromosomes should be of normal appearance. However,
3. in the third case (colorless, waxy), where crossing over had occurred, one would hope to find evidence that a physical exchange of parts between the homologous chromosomes of the dihybrid parent had occurred. Either a chromosome of normal length, but with a knob at one end, or an extra-long chromosome with no knob should be present. Creighton and McClintock found the latter, thus indicating that the gene locus for ***wx*** was associated with (and thus near) the end of the chromosome with the extra segment. The gene locus for kernel color must then be neared the end with the knob.
4. Examination of the plants in class 4 (colored kernels and normal endosperm) revealed a chromosome of normal length but with a knob at the end.

Thus, behavior of the genes as revealed by the study of the phenotypes produced was shown to be directly related to the behavior of chromosomes as seen under the microscope. The recombination of genes occurs when homologous chromosomes exchange parts.

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8.4: Genetic linkage and Genetic Maps

Background

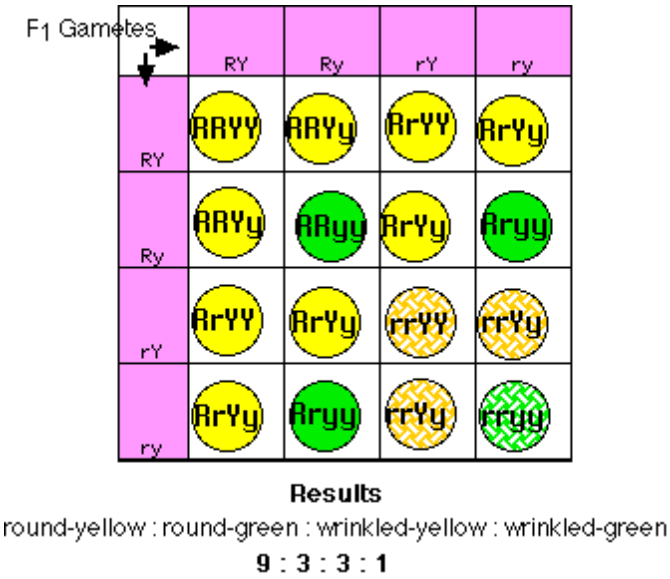


Figure 8.4.1 Dihybrid Cross

Mendel then crossed these dihybrids. If it is inevitable that round seeds must always be yellow and wrinkled seeds must be green, then he would have expected that this would produce a typical **monohybrid** cross: 75% round-yellow; 25% wrinkled-green. But, in fact, his mating generated seeds that showed all possible combinations of the color and texture traits.

- 9/16 of the offspring were round-yellow
- 3/16 were round-green
- 3/16 were wrinkled-yellow, and
- 1/16 were wrinkled-green

Rule of Independent Assortment

Finding in every case that each of his seven traits was inherited independently of the others, he formed his "second rule" the **Rule of Independent Assortment**:

> The inheritance of one pair of factors (genes) is independent of the inheritance of the other pair.

Today we know that this rule holds only if two conditions are met:

- the genes are on separate chromosomes or
- the genes are widely separated on the same chromosome.

Mendel was lucky in that every pair of genes he studied met one requirement or the other. The table shows the chromosome assignments of the seven pairs of alleles that Mendel studied. Although all of these genes showed independent assortment, several were, in fact, **syntenic** with three loci occurring on chromosome 4 and two on chromosome 1. However, the distance separating the syntenic loci was sufficiently great that the genes were inherited as though they were on separate chromosomes.

Trait	Phenotype	Alleles	Chromosome
Seed form	round-wrinkled	*R-r*	7
Seed color	yellow-green	*I-i*	1
Pod color	green-yellow	*Gp-gp*	5
Pod texture	smooth-wrinkled	*V-v*	4
Flower color	purple-white	*A-a*	1
Flower location	axial-terminal	*Fa-fa*	4
Plant height	tall-dwarf	*Le-le*	4

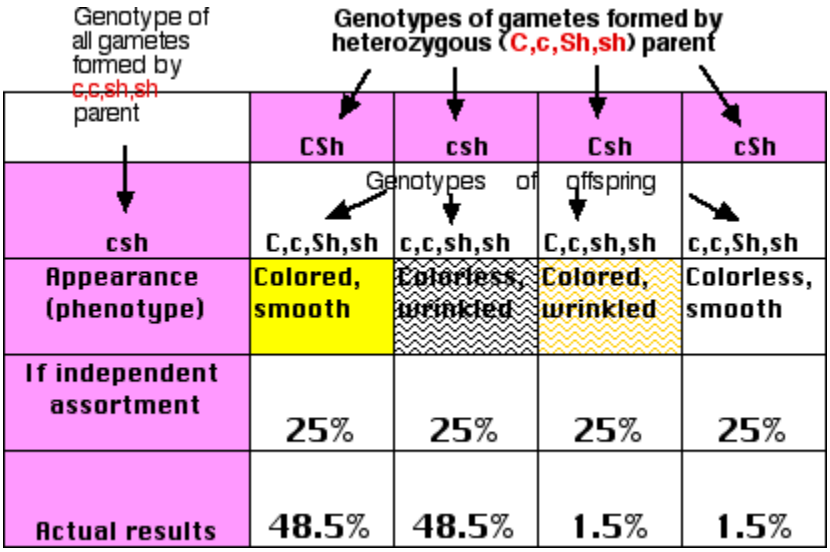


Figure 8.4.2. Linkage in corn

Start with two different strains of corn (maize).

- one that is homozygous for two traits
  - yellow kernels (**C**,**C**) which are filled with endosperm causing the kernels to be
  - smooth (**Sh**,**Sh**).
- a second that is homozygous for
  - colorless kernels (**c**,**c**) that are wrinkled because their endosperm is
  - shrunken (**sh**,**sh**)

When the pollen of the first strain is dusted on the silks of the second (or vice versa), the kernels produced (F_1) are all yellow and smooth. So the alleles for yellow color (**C**) and smoothness (**Sh**) are dominant over those for colorlessness (**c**) and shrunken endosperm (**sh**).

To simplify the analysis, mate the dihybrid with a **homozygous recessive** strain (**ccshsh**). Such a mating is called a **test cross** because it exposes the genotype of all the gametes of the strain being evaluated.

According to Mendel's second rule, the genes determining color of the endosperm should be inherited independently of the genes determining texture. The F_1 should thus produce gametes in approximately equal numbers.

- CSh, as inherited from one parent.
- csh, as inherited from the other parent
- Csh, a recombinant
- cSh, the other recombinant.

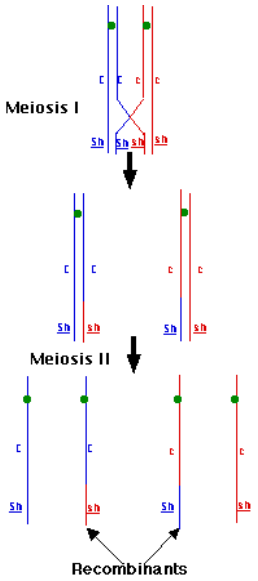


Figure 8.4.4 Plot Linkage

In fact, the recombination frequency is 2.0%, telling us that the actual order of loci is **c — sh — bz**.

Mapping by linkage analysis is best done with loci that are relatively close together; that is, within a few centimorgans of each other. Why? Because as the distance between two loci increases, the probability of a second crossover occurring between them also increases.

But a second crossover would undo the effect of the first and **restore the parental combination** of alleles. These would show up as nonrecombinants. Thus as the distance between two loci increases, the percentage of recombinants that forms **understates** the actual distance in centimorgans that separates them. And, in fact, that has happened in this example. Using a **three-point cross** reveals the existence of a small number of double recombinants and tells us that the actual distance c—bz is indeed 5 cM as we would expect by summing

- c—sh = 3 cM
- sh—bz = 2 cM

and not the 4.6 cM revealed by the dihybrid cross.

A three-point cross also tells us the gene order in a single cross rather than the three we needed here.

There are other problems with preparing genetic maps of chromosomes.

- The probability of a crossover is not uniform along the entire length of the chromosome.
  - Crossing over is inhibited in some regions (e.g., near the centromere).
  - Some regions are "hot spots" for recombination (for reasons that are not clear). Approximately 80% of genetic recombination in humans is confined to just one-quarter of our genome.
- In humans, the frequency of recombination of loci on most chromosomes is higher in females than in males. Therefore, genetic maps of female chromosomes are longer than those for males.

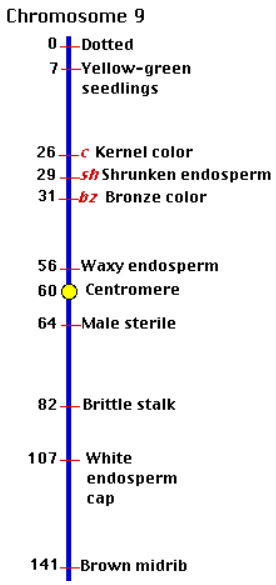


Figure 8.4.5 Genetic Map of Chromosome 9

A genetic map of chromosome 9 (the one that carries the **C**, **Sh**, and **bz** loci) of the corn plant (***Zea mays***) is shown above. If one maps in small intervals from one end of a chromosome to the other, the total number of centimorgans often exceeds 100 (as you can see for chromosome 9). However, even for widely-separated loci, the maximum frequency of recombinants that can form is 50%. And this is also the frequency of recombinants that we see for genes independently assorting on separate chromosomes. So we cannot tell by simply counting recombinants whether a pair of gene loci is located far apart on the same chromosome or are on different chromosomes. As we saw above, several of Mendel's independently assorting traits are controlled by genes on the same chromosome but located so far apart that they are inherited as if they were located on different chromosomes.

Genes that are present on the same chromosome are called **syntenic**.

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8.5: Gene Mapping with Three-point Crosses

Mechanisms discussed previously show how carrying out **three** different **dihybrid test crosses** in the corn plant reveals the order of the gene loci and the distance between them (in centimorgans, **cM**). Here we shall see how a **single** test cross of a **trihybrid** corn plant; that is, one parent is heterozygous for three linked alleles (C,Sh, Bz, on one chromosome; c,sh,bz on the other) and the other parent is homozygous for the recessive version of all three genes (c,c,sh,sh,bz,bz) reveals the gene order and gives a more accurate measurement of the distance in cM separating the outermost loci (in this case **C** and **Bz**) than a dihybrid cross involving those loci would. Hypothetical breeding data are shown in Table 8.5.1.

Table 8.5.1: Hypothetical breeding data

Group	Expressed Alleles (Phenotype)	Crossovers	Number	Totals
1	**CShBz**	None; the parentals	479	952
2	**cshbz**		473	
3	**C|shbz**	Single; between C and others	15	28
4	**c|ShBz**		13	
5	**CSh|bz**	Single, between Bz and others	9	18
6	**csh|Bz**		9	
7	**C|sh|Bz**	Double recombinants	1	2
8	**c|Sh|bz**		1	
		Totals	1000	

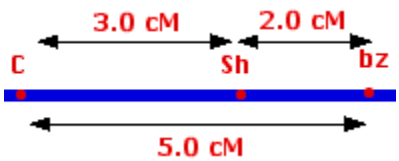
Eight different **phenotypes** — representing the 8 possible genotypes (2^3 = 8) are produced. Scoring them reveals

- The percentage of recombinants between **C** and **Sh** is 3.0%: 28/1000 of single recombinants **plus** 2/1000 double recombinants.
- That between **Sh** and **Bz** is 2.0%: 18/1000 single recombinants **plus** 2/1000 double recombinants.
- That between **C** and **Bz** is 4.6%: 28 + 18 = 46/1000.

But adding the distances between **C** and **Sh** and **Sh** and **Bz** gives a map distance between **C** and **Bz** of 5.0 cM not the 4.6 cM revealed by the data (and the same number that a C,c,Bz,bz **dihybrid cross** would have produced). Why the discrepancy? Because the double recombinants restored the parental configuration, they were missed in the scoring. So the two rare classes of double recombinants need to be added (twice) to the data.

$$28 + 18 + 2 + 2 = 50/1000 = 5\% \tag{8.5.1}$$

to get the true value. So the map of this region of the chromosome is:



This exercise underscores the rule that the closer the intervals examined, the more accurate the map. A three-point cross also gives the **gene order** immediately. The procedure is:

1. Determine the **rarest** classes (here, C,sh,Bz and c,Sh,bz) because two crossovers between a pair of loci will be rarer than one.
2. In these two groups, the alleles that specify the trait that was **not** seen in the parents (sh and Sh) occupy the **middle** locus.

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8.6: Quantitative Trait Loci

The rules of inheritance discovered by Mendel depended on his wisely choosing traits that varied in a clear-cut, easily distinguishable, **qualitative** way. But humans are not either tall or short nor are they either heavy or light. Many traits differ in a continuous, **quantitative** way throughout a population.

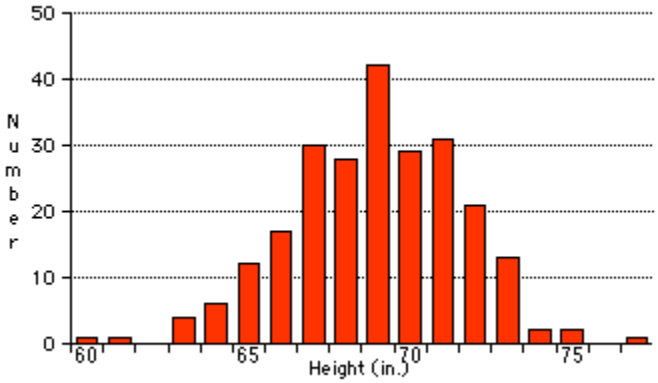


Figure 8.6.1: Distribution of heights among group of male secondary-school seniors

This histogram shows the distribution of heights among a group of male secondary-school seniors. As you can see, the plot resembles a bell-shaped curve. Such distributions are typical of quantitative traits. Some of the variation can be explained by differences in diet and perhaps other factors in the environment. Environment alone is not, however, sufficient to explain the full range of heights or weights.

An understanding of how genes can control quantitative traits emerged in 1908 from the work of the Swedish geneticist Nilsson-Ehle who studied quantitative traits in wheat. Using Mendel's methods, he mated pure-breeding red-kernel strains with pure-breeding white-kernel strains. The offspring were all red, but the intensity of color was much less that in the red parent. It seemed as though the effect of the red allele in the F_1 generation was being modified by the presence of the white allele.

Nilsson-Ehle: Genetics of Two Crosses

When Nilsson-Ehle mated two F_1 plants, he produced an F_2 generation in which red-kerneled plants outnumbered white-kerneled plants 15:1. But the red kernels were not all alike. They could quite easily be sorted into four categories. One sixteenth of them were deep red, like the *P* type. Four sixteenths were medium dark red, six sixteenths were medium red (like the F_1 generation), and four sixteenths were light red. The genetics of the two crosses is shown here. The alleles at one locus are indicated with prime marks; at the other, without.

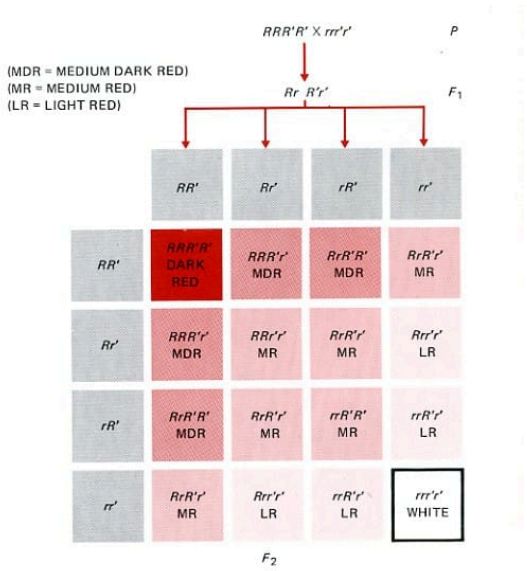


Figure 8.6.2: Genetics of the two crosses by Nilsson-Ehle

These results could be explained by assuming that kernel color in wheat is controlled by not one, but two pairs of genes, the effects of which add up without distinct dominance. Each pair is located on a different chromosome or so far apart on the same chromosome that there is no linkage. Four alleles for red produce a deep red kernel. Four alleles for white produce a white kernel. Just one red allele out of four produces a light red kernel. Any two out of the four produce a medium red kernel. Any three of the four produce a medium dark red kernel. If one plots the numbers of the different colored offspring in the F_2 generation against color intensity, one gets a graph like Figure 8.6.3.

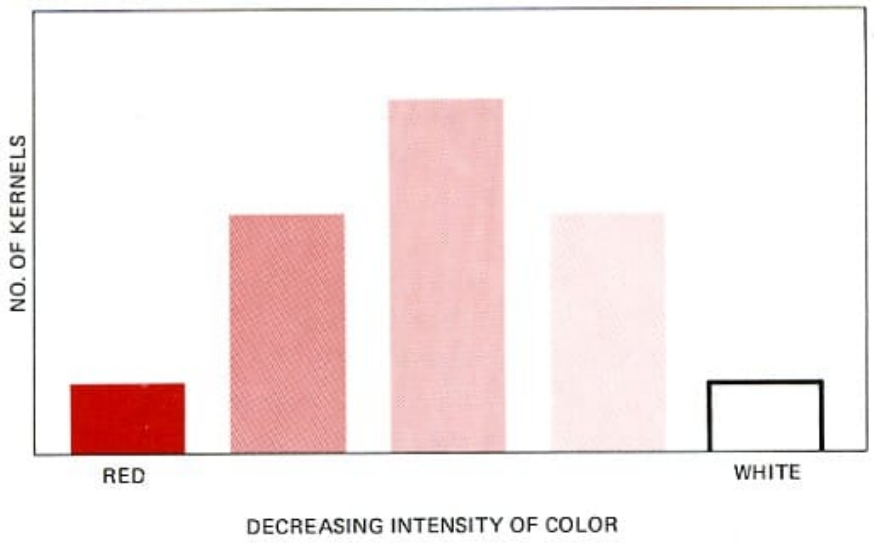


Figure 8.6.3: Genetics of the two crosses results interpretation by Nilsson-Ehle,

In other wheat varieties, Nilsson-Ehle found F_2 generations with a ratio of red kernels to white of 63:1. These could be explained by assuming that three pairs of alleles were involved. In these cases, six different shades of red could be detected, but the color differences were very slight. Environmental influences also caused alterations in intensity so that in practice the collection of kernels displayed a continuous range of hues all the way from deep red to white.

So the occurrence of continuous variation of a trait in a population can be explained by assuming it is controlled by several pairs of genes — called **quantitative trait loci** (**QTL**) — the effects of which are added together. This is called **polygenic inheritance** or the **multiple-factor hypothesis**. At first the study of quantitative traits was mostly confined to animal husbandry and the breeding of agricultural crops. It was based on the premise that

- When two extreme types ae mated (e.g., *AABB* and *aabb*). the offspring are intermediate in type.
- When two intermediate types are mated, most of their offspring are also intermediate, but some extreme types will be produced.
- The results of random matings in a large population will be a large range of types with the greatest number in the middle range and the fewest at the extremes.

In more recent times, the search for quantitative trait loci has turned to humans. A number of diseases, cancers for example, are thought to be caused by the additive effects of genes at different loci. Pedigree analysis has provided some insights, but the use of microarrays promises to provide more.

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8.7: Mapping the Genes of T2

T2 and its close relative **T4** are viruses that infect the bacterium *E. coli*. The infection ends with destruction (**lysis**) of the bacterial cell so these viruses are examples of bacteriophages ("bacteria eaters"). Each virus particle (**virion**) consists of:

- a protein head (~0.1 µm) inside of which is a single, circular molecule of double-stranded DNA containing 166,000 base pairs.
- a protein tail from which extend
- thin protein fibers

Life Cycle

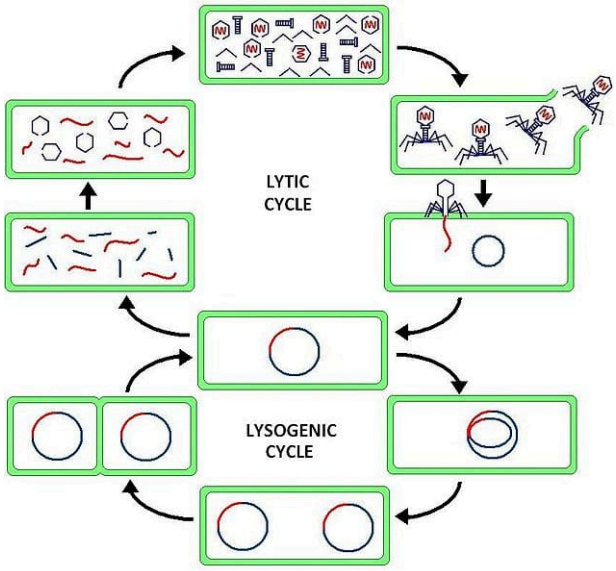


Figure 8.7.1: T4: bacteriophage lysogenic and lytic cycle. (CC BY-SA 3.0; Suly12)

The virus attaches to the *E. coli* cell (**a**). This requires a precise molecular interaction between the fibers and the cell wall of the host.

- The DNA molecule is injected into the cell (**b**).
- Within 1 minute, the viral DNA begins to be transcribed and translated into some of the viral proteins, and synthesis of host proteins is stopped.
- At 5 minutes, viral enzymes needed for synthesis of new viral DNA molecules are produced (**c**).
- At 8 minutes, some 40 different structural proteins for the viral head and tail are synthesized.
- At 13 minutes, assembly of new viral particles begins (**d**).
- At 25 minutes, the viral lysozyme destroys the bacterial cell wall and the viruses burst out — ready to infect new hosts (**e**).
    - If the bacterial cells are growing in liquid culture, it turns clear.
    - If the bacterial cells are growing in a "lawn" on the surface of an agar plate, then holes, called **plaques**, appear in the lawn.

Occasionally, new phenotypes appear such as a change in the appearance of the plaques or even a loss in the ability to infect the host.

Examples:

- ***h***
    - Some strains of *E. coli*, e.g. one designated B/2, gain the ability to resist infection by normal ("wild-type") T2. The mutation has caused a change in the structure of their cell wall so that the tail fibers of T2 can no longer bind to it. However, T2 can strike back. Occasional T2 mutants appear that overcome this resistance. The mutated gene, designated *h* (for

"host range"), encodes a change in the tail fibers so they can once again bind to the cell wall of strain B/2. The normal or "wild-type" gene is designated h^+ .
    - When plated on a lawn containing both *E. coli* **B** and *E. coli* **B/2**,
        - the mutant (*h*) viruses can lyze both strains of *E. coli*, producing clear plaques, while
        - the wild-type (h^+) viruses can only lyze *E. coli* B producing mottled or **turbid plaques**.
- ***r***
    - Occasional T2 mutants appear that break out of their host cell earlier than normal.
    - The mutation occurs in a gene designated *r* (for "rapid lysis"). It reveals itself by the extra-large plaques that it forms.
    - The wild-type gene, producing a normal time of lysis, is designated r^+. It forms normal-size plaques.

As with so many organisms, the occurrence of mutations provides the tools to learn about such things as

- the function of the gene
- its location in the DNA molecule (mapping)

Mapping by Recombination Frequencies

As we have seen, *E. coli* strain B can be infected by both h^+ and *h* strains of T2. In fact, a single bacterial cell can be infected simultaneously by both. Let us infect a liquid culture of *E. coli* **B** with two different mutant T2 viruses: *h* r^+ and h^+ r. When this is done in liquid culture, and then plated on a mixed lawn of *E. coli* **B** and **B/2**, four different kinds of plaques appear.

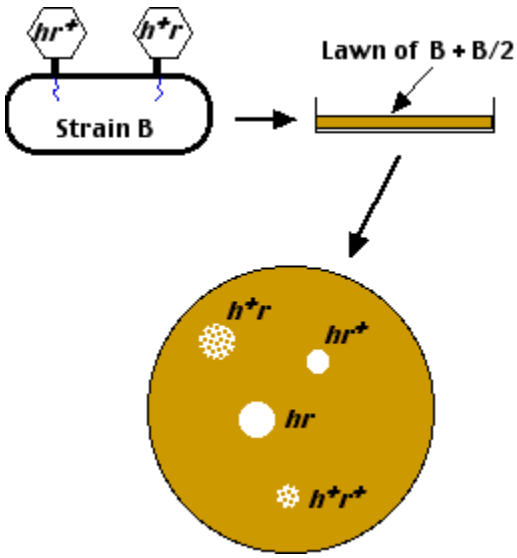
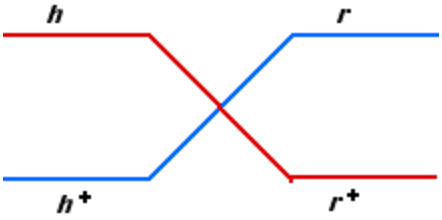


Figure 8.7.2 E. Coli B mapping

Genotype	Phenotype	Number of Plaques
hr^+	clear, small	460
h^+r	turbid, large	460
h^+r^+	turbid, small	40
hr	clear, large	40
	Total =	1000

The most abundant (460 each) are those representing the parental types; that is, the phenotypes are those expected from the two infecting strains. However, small numbers (40 each) of two new phenotypes appear. These can be explained by **genetic recombination** having occasionally occurred between the DNA of each parental type within the bacterial cell.



Recombination frequency = 0.08
Therefore, *h* and *r* separated by 8 map units (centimorgans, cM)

Just as in higher organisms, one assumes that the frequency of recombinants is proportional to the distance between the gene loci. In this case, 80 out of 1000 plaques were recombinant, so the distance between the *h* and *r* loci is assigned a value of 8 **map units** or **centimorgans** (cM). Now coinfect *E. coli* **B** with two other strains of T2:

- hm^+ and
- h^+m

hm^+	470
h^+m	470
h^+m^+	30
hm	30
Total =	1000

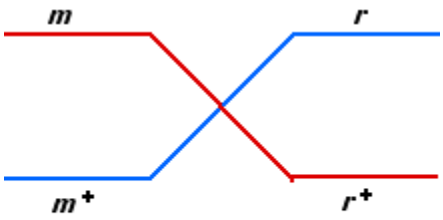
Again, 4 kinds of plaques are produced: parental (470 each) and recombinant (30 each).

The smaller number of recombinants indicates that these two gene loci (*h* and *m*) are closer together (6 cM) than *h* and *r* (8 cM). But the order of the three loci could be either

- *m*–6–*h*—8—*r*
  or
- *h*–6–*m*-2-*r*

To find out which is the correct order, perform a third mating using

- mr^+ and
- m^+r



Recombination frequency = 0.12
Therefore, *m* and *r* separated by 12 map units (centimorgans, cM)

mr^+	440

m^+r	440
m^+r^+	60
mr	60
Total =	1000

This makes it clear that the order is m—h—r, not h—m—r.

But why only 12 cM between the outside loci (*m* and *r*) instead of the 14 cM produced by adding the map distances found in the first two matings?

A Three-Point Cross

The answer comes from performing a mating between T2 viruses differing at all three loci:

- *hmr*
  and
- $h^+m^+r^+$

(Note: this time one parent has all mutant; the other all wild-type alleles — don't be confused!)

Group 1	*hmr*	435
Group 2	$h^+m^+r^+$	435
Group 3	h^+mr^+	25
Group 4	hm^+r	25
Group 5	hmr^+	35
Group 6	h^+m^+r	35
Group 7	hm^+r^+	5
Group 8	h^+mr	5
	Total =	1000

The result: 8 different types of plaques are formed.

- parentals; that is, nonrecombinants in Groups 1 and 2;
- recombinants — all the others

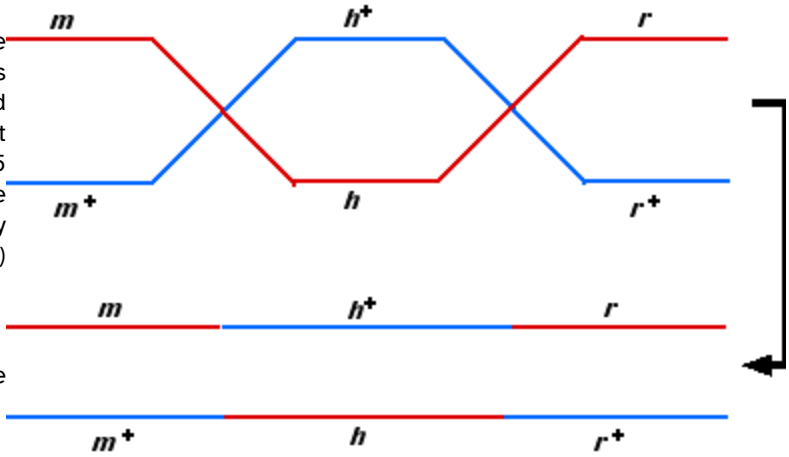
Analyzing these data shows how the **two**-point cross between *m* and *r* **understated** the true distance between them.

Let's first look at single pairs of recombinants as we did before (thus ignoring the third locus).

- If we look at all the recombinants between *h* and *r* but **ignore** *m* (as in the first experiment), we find that they are contained in Groups 5, 6, 7, and 8 — giving the total of 80 that we found originally.
- If we look at recombinants between *h* and *m* but ignore *r* (as in the second experiment), we find that they are contained in Groups 3, 4,7, and 8 — giving the same total of 60 that we found before.
- But if we focus **only** on *m* and *r* (as we did in the third experiment), we find that the recombinants are contained in Groups 3, 4, 5, and 6 — giving the same total of 120 as before while the non-recombinants are not only in Groups 1 and 2 but also in Groups 7 and 8. The reason: a double-crossover occurred in these cases, restoring the **parental configuration** of the *m* and

r alleles.

- Because these double crossovers were hidden in the third experiment, the map distance (12 cM) was understated. To get the true map distance, we add their number to each of the other recombinant groups (Groups 3,4,5, and 6) so 25 + 5 +25 +5 +35 + 5 + 35 + 5 = 140, and the true map distance between *m* and *r* is the 14 cM that we found by adding the map distances between *h* and *r* (8 cM) and *h* and *m* (6 cM).



The three-point cross is also useful because it gives the gene order simply by inspection:

- Find the rarest genotypes (here Groups 7 and 8)
- The gene NOT in the parental configuration (here *h*) is always the middle one.

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8.8: rII Locus of T4

Mapping Within A Gene: the RII Locus

T2 and its close relative **T4** are viruses that infect the bacterium *E. coli*. The infection ends with destruction (**lysis**) of the bacterial cell so these viruses are examples of bacteriophages ("bacteria eaters"). They have been enormously useful in genetic studies because:

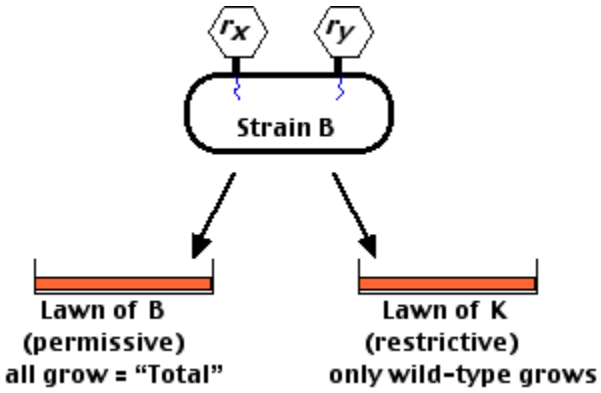
- Viruses of two (or more) different genotypes can simultaneously infect a single bacterium.
- The DNA molecules of one of the infecting viruses can recombine with that of another forming recombinant molecules.
- The huge number of viruses released from a huge number of bacterial hosts enables even rare recombination events to be detected.

Another page — Bacteriophage Genetics — describes how T2 is used to map the order and relative spacing of genes on the single circular molecule of DNA that is the virus's genome. Here let us see how T4 can be used to detect mutations within a single gene and speed up the process of mapping these point mutations by the use of **deletion mutants**.

Detecting Mutation within a Single Gene

In Bacteriophage Genetics, we examined mutation of a gene designated *r*, for "rapid lysis". It turned out that actually there are three different gene **loci** — *rI*, *rII*, and *rIII* — mutations in any one of which produced a rapid-lysis phenotype. But, in addition, there were many mutations found in each of these. Could wild-type virus be formed by recombination between mutations within the same gene? Seymour Benzer decided to find out.

As we saw in Bacteriophage Genetics, the recombination frequency between **different** genes is low (on the order of 10^{-2}). One would expect that recombination frequencies between mutations in a single gene would be far lower (10^{-4} or less). Fortunately Benzer could exploit a phenomenon to enable him to detect such rare events: *rII* **mutants** as well as wild-type T4 can infect and complete their life cycle in a strain of *E. coli* designated B. However, while rII mutants can infect a strain of E. coli designated K, they cannot complete their life cycle in strain K. Wild-type T4 can.



The procedure was to infect strain B in liquid culture with two mutants to be tested (designated here as r_X and r_Y). After incubation, these were plated on a lawn of:

- strain B — which supports the growth of all viruses thus giving the total number of viruses liberated.
- strain K — on which only wild-type viruses can grow.

The recombination frequency between any pair of mutations is calculated as

$$\text{Recombination Frequency} = \frac{2 \times \text{number of wild-type plaques (strain K plaques)}}{\text{total number of plaques (on strain B)}} \tag{8.8.1}$$

You have to double the number found on strain K because you only see one-half the recombinants — the other half consists of double mutants. Using this technique, Benzer eventually found some 2000 different mutations in the rII gene. The recombination frequency between some pairs of these was as low as 0.02.

- The T4 genome has 160,000 base pairs of DNA extending over ~1,600 centimorgans (cM).
- So 1 cM ≅ 100 base pairs
- So 0.02 cM represents a pair of adjacent nucleotides.
- From these data, Benzer concluded that the
  - smallest unit of mutation and
  - the smallest unit of recombination

  was a single base pair of DNA.

In other words, these mutations represent a change in a single base pair — we call these point mutations. Recombination between two molecules of DNA can occur at any pair of nucleotides.

Mapping Point Mutations Within A Gene

The relative order and spacing of any two point mutations in a single gene like *rII* can be done using the procedure describe in Bacteriophage Genetics. But with some 2000 different mutations to test, the process would be tremendously time-consuming. (Even using the procedure to be described now, Benzer spent some 10 years on the project.) Benzer was able to speed up the mapping process by taking advantage of the discovery that some of his mutants did not have point mutations but deletions instead. In contrast to the properties of T4 viruses with point mutations, T4 viruses with deletions in *rII* showed no recombination with other *rII* mutants or any other genes for that matter. Moreover, these deletions never back-mutated.

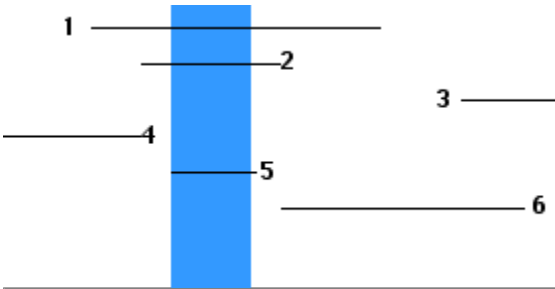
Deletion Mapping

Deletions can be mapped by the same procedure used for point mutations. Simply cross pairs of deletion mutants and see if they produce progeny that can grow on *E. coli* strain K. Here is a hypothetical example. Each of 6 strains of deletion mutants are crossed with each of the others.

Table 8.8.1:

Strains	1	2	3	4	5	6	
1	0	0	+	0	0	0	1 and 3 do not overlap
2		0	+	+	0	0	must shift 4 away from 2
3			0	+	+	0	6 must extend under 3
4				0	+	+	right-hand end of 4 must be removed from over 6
5					0	+	left-hand end of 6 must not overlap 5 but must continue to overlap 2. ∴ shorten right-hand end of 5
6						0	

From the results, one can draw a map showing the order and relative size of the deletions.



With such a deletion map, one can now quickly map the location of **point mutations** by coinfecting each of the different deletion strains (here 1–6) with the mutant strain ("x").

There is no longer any need to count plaques; simple see whether there is growth or not.

Coinfect with strain	1	2	3	4	5	6

and mutant "x" Results →	0	0	+	+	0	+

From these results, we learn that the point mutation "x" is located on the T4 DNA within the region shown above in blue.

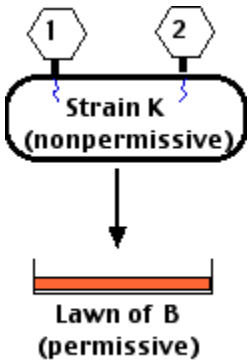
Complementation

As we saw above, rapid lysis (*r*) mutants were found that mapped to three different regions of the T4 genome: *rI*, *rII*, and *rIII*. This meant that those in different regions were not alleles of the same gene and more than one gene product participated in the lysis function. Even within one "locus", **rII**, there turned out to be two different stretches of DNA both of which were needed intact for the lysis function. This was revealed by the complementation test that Benzer used. In this test,

- *E. coli* strain K (which *rII* mutants can infect but not complete their life cycle) — growing in liquid culture — was
- coinfected with two different *rII* mutants (shown in the figure as "1" and "2").

Note that this procedure differs from the earlier one (recombination) in that the nonpermissive *E. coli* **K** is used for the initial infection (not strain B as before). Neither strain *rII*"1" nor strain *rII*"2" is able to grown in *E. coli* K. But if the lost function in *rII*"1" is NOT the same as the lost function in *rII*"2", then

- each should be able to produce the gene product missing in the other — **complementation** — and
- living phages will be produced. (Again, there is no need to count plaques; simply see if they are formed or not.)



Mutant strains	1	2	3	4	5
1	0	0	+	0	+
2		0	+	0	+
3			0	+	0
4				0	+
5					0

From these results, you can deduce that these 5 *rII* mutants fall into two different complementation groups, which Benzer designated A (containing strains 1, 2, and 4) and B (containing strains 3 and 5). Later work showed that the function of *rII* depended on the polypeptide products encoded by two adjacent regions (A and B) of *rII* (perhaps acting as a heterodimer). In terms of function, then, both A and B qualify as independent genes. In coinfections by two mutant strains,

- If either A or B is mutated on the same DNA molecule ("*cis*"), there is no function while
- if A is mutated in one DNA molecule and B in the other ("trans"), function is restored.

Complementation, then, is the ability of two different mutations to restore wild-type function when they are in the "trans" (on different DNA molecules), but not when they are in "cis" (on the same DNA molecule). Benzer coined the term **cistron** for these genetic units of function. But today, we simply modify earlier concepts of the "gene" to fit this operational definition.

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UNIT 9: REGULATION OF GENE EXPRESSION

9.1: Regulation of Gene Expression in Bacteria

9.2: The Tryptophan Repressor

9.3: Regulation of Gene Expression in Eukaryotes

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9.1: Regulation of Gene Expression in Bacteria

The Operon

Within its tiny cell, the bacterium *E. coli* contains all the genetic information it needs to metabolize, grow, and reproduce. It can synthesize every organic molecule it needs from glucose and a number of inorganic ions. Many of the genes in *E. coli* are expressed constitutively; that is, they are always turned "on". Others, however, are active only when their products are needed by the cell, so their expression must be regulated.

Two examples:

- If the amino acid tryptophan (**Trp**) is added to the culture, the bacteria soon stop producing the five enzymes previously needed to synthesize Trp from intermediates produced during the respiration of glucose. In this case, the presence of the products of enzyme action **represses** enzyme synthesis.
- Conversely, adding a new substrate to the culture medium may **induce** the formation of new enzymes capable of metabolizing that substrate. If we take a culture of *E. coli* that is feeding on glucose and transfer some of the cells to a medium contain lactose instead, a revealing sequence of events takes place.
    - At first the cells are quiescent: they do not metabolize the lactose, their other metabolic activities decline, and cell division ceases.
    - Soon, however, the culture begins growing rapidly again with the lactose being rapidly consumed. What has happened? During the quiescent interval, the cells began to produce **three enzymes**.

The three enzymes are

- a **permease** that transports lactose across the plasma membrane from the culture medium into the interior of the cell
- **beta-galactosidase** which converts lactose into the intermediate allolactose and then hydrolyzes this into glucose and galactose. Once in the presence of lactose, the quantity of beta-galactosidase in the cells rises from a tiny amount to almost 2% of the weight of the cell.
- a **transacetylase** whose function is still uncertain.

The *lac* operon

The capacity to respond to the presence of lactose was always there. The genes for the three induced enzymes are part of the genome of the cell. But until lactose was added to the culture medium, these genes were not expressed (β-galactosidase was expressed weakly — just enough to convert lactose into allolactose). The most direct way to control the expression of a gene is to **regulate its rate of transcription**; that is, the rate at which RNA polymerase transcribes the gene into molecules of messenger RNA (mRNA).

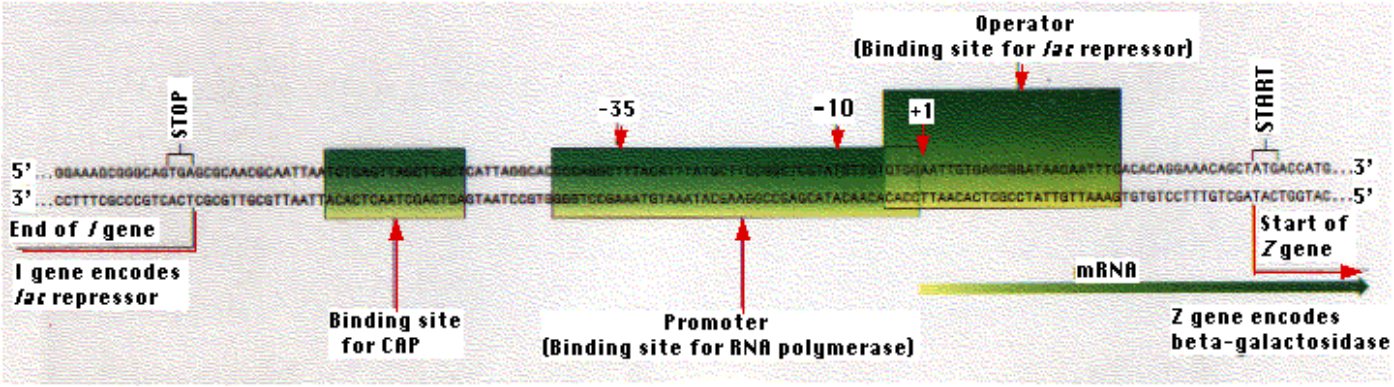


Figure 9.1.1 The lac DNA transciprtion

Gene transcription begins at a particular nucleotide shown in the figure as "**+1**". RNA polymerase actually binds to a site "upstream" (i.e., on the 5' side) of this site and opens the double helix so that transcription of one strand can begin. The binding

site for RNA polymerase is called the promoter. In bacteria, two features of the promoter appear to be important:

- a sequence of TATAAT (or something similar) centered 10 nucleotides upstream of the **+1** site and
- another sequence (TTGACA or something quite close to it) centered 35 nucleotides upstream.

The exact DNA sequence between the two regions does not seem to be important. Each of the three enzymes synthesized in response to lactose is encoded by a separate gene. The three genes are arranged in tandem on the bacterial chromosome.

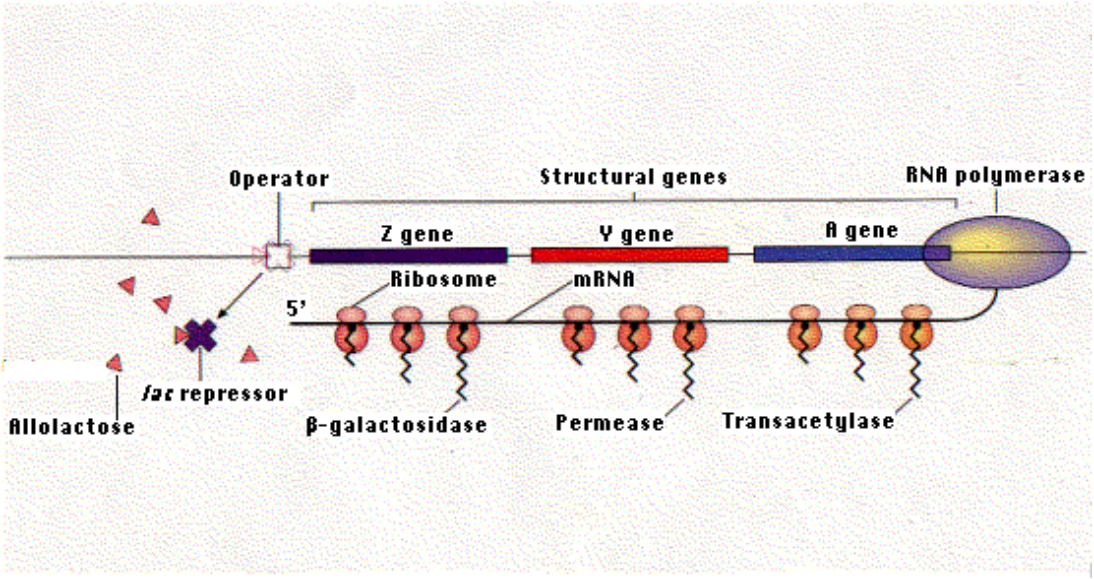


Figure 9.1.2 The lac Operon

In the absence of lactose, the repressor protein encoded by the I gene binds to the *lac* operator and prevents transcription. Binding of allolactose to the repressor causes it to leave the operator. This enables RNA polymerase to transcribe the three genes of the operon. The single mRNA molecule that results is then translated into the three proteins.

The ***lac* repressor** binds to a specific sequence of two dozen nucleotides called the **operator**. Most of the operator is downstream of the promoter. When the repressor is bound to the operator, RNA polymerase is unable to proceed downstream with its task of gene transcription. The lac repressor represents only a tiny fraction of the proteins in the *E. coli* cell.

The operon is the combination of the operator and the three protein-encoding genes associated with it.

The gene encoding the *lac* repressor is called the *I* gene. It happens to be located just upstream of the *lac* promoter. However, its precise location is probably not important because it achieves its effect by means of its protein product, which is free to diffuse throughout the cell. And, in fact, the genes for some repressors are not located close to the operators they control.

Although repressors are free to diffuse through the cell, how does — for example — the lac repressor find the single stretch of 24 base pairs of the operator out of the 4.6 million base pairs of DNA in the *E. coli* genome? It turns out the repressor is free to bind **anywhere** on the DNA using both

- hydrogen bonds and
- ionic (electrostatic) interactions between its positively-charged amino acids (Lys, Arg) and the negative charges on the deoxyribose-phosphate backbone of the DNA.

Once astride the DNA, the repressor can move along it until it encounters the operator sequence. Now an allosteric change in the tertiary structure of the protein allows the same amino acids to establish bonds — mostly hydrogen bonds and hydrophobic interactions — with particular bases in the operator sequence.

The *lac* repressor is made up of four identical polypeptides (thus a "homotetramer"). Part of the molecule has a site (or sites) that enable it to recognize and bind to the 24 base pairs of the lac operator. Another part of the repressor contains sites that bind to allolactose. When allolactose unites with the repressor, it causes a change in the shape of the molecule, so that it can no longer remain attached to the DNA sequence of the operator. Thus, when lactose is added to the culture medium, it causes

the repressor to be released from the operator and RNA polymerase can now begin transcribing the 3 genes of the operon into a **single** molecule of **messenger RNA**.

Hardly does transcription begin, before ribosomes attach to the growing mRNA molecule and move down it to **translate** the message into the three proteins. You can see why punctuation codons — UAA, UAG, or UGA — are needed to terminate translation between the portions of the mRNA coding for each of the three enzymes. This mechanism is characteristic of bacteria, but differs in several respects from that found in eukaryotes:

- Genes in eukaryotes are not linked in operons (except for nematodes like *C. elegans* and tunicates like *Ciona intestinalis*).
- Primary transcripts in eukaryotes contain the transcript of only a single gene (with the above exceptions).
- Transcription and translation are not physically linked in eukaryotes as they are in bacteria; transcription occurs in the nucleus while translation occurs in the cytosol (with a few exceptions).

C. elegans

C. elegans differs from most eukaryotes in having a substantial fraction (15–20%) of its genes grouped in operons containing from 2 to 8 genes each. Like bacteria, all the genes in an operon are transcribed from a single promoter producing a single primary transcript (pre-mRNA). Some of the genes in these operons appear — as in bacteria — to be involved in the same biochemical function, but this may not be the case for most. *C. elegans* operons also differ from those in bacteria in that each pre-mRNA is processed into a separate mRNA for each gene rather than being translated as a unit.

Corepressors

As mentioned above, the synthesis of tryptophan from precursors available in the cell requires 5 enzymes. The genes encoding these are clustered together in a single operon with its own promoter and operator. In this case, however, the **presence** of tryptophan in the cell **shuts down** the operon. When Trp is present, it binds to a site on the Trp repressor and **enables** the Trp repressor to bind to the operator. When Trp is not present, the repressor leaves its operator, and transcription of the 5 enzyme-encoding genes begins.

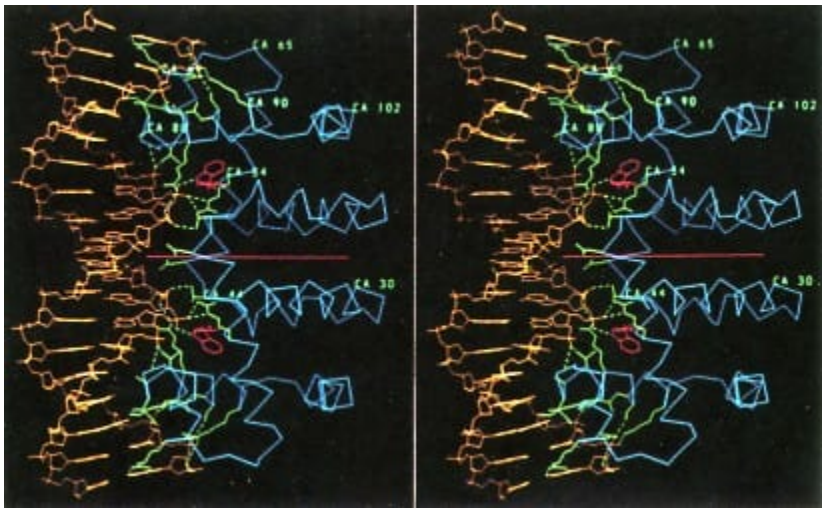


Figure 9.1.3: Tryptophan Repressor courtesy of P. B. Sigler

The above picure shows stereo view of the tryptophan repressor (right side of each panel) bound to its operator DNA (left side). The repressor is a homodimer of two identical polypeptides (on either side of the horizontal red line). Binding to DNA occurs only when a molecule of tryptophan (red rings) is bound to each monomer of the repressor. The usefulness to the cell of this control mechanism is clear. The presence in the cell of an essential metabolite, in this case tryptophan, turns off its own manufacture and thus stops unneeded protein synthesis. As its name suggests, repressors are **negative control** mechanisms, shutting down operons

- in the absence of a substrate (lactose in our example) or
- the presence of an essential metabolite (tryptophan is our example).

However, some gene transcription in *E. coli* is under positive control.

Positive Control of Transcription: CAP

Absence of the lac repressor is essential but not sufficient for effective transcription of the lac operon. The activity of RNA polymerase also depends on the presence of another DNA-binding protein called **catabolite activator protein (CAP)**. Like the lac repressor, CAP has two types of binding sites: One binds the nucleotide cyclic AMP and the other binds a sequence of 16 base pairs upstream of the promoter

However, CAP can bind to DNA only when cAMP is bound to CAP. so when cAMP levels in the cell are low, CAP fails to bind DNA and thus RNA polymerase cannot begin its work, even in the absence of the repressor. So the *lac* operon is under both **negative** (the **repressor**) and **positive** (**CAP**) control. Why?

It turns out that it is not simply a matter of belt and suspenders. This dual system enables the cell to **make choices**. What, for example, should the cell do when fed both glucose and lactose? Presented with such a choice, *E. coli* (for reasons about which we can only speculate) chooses glucose. It makes its choice by using the interplay between these two control devices.

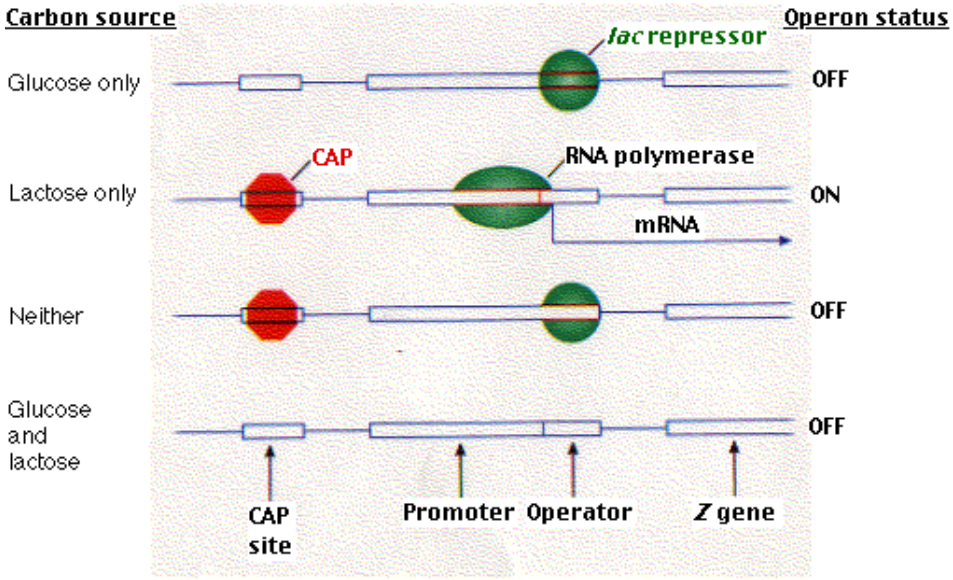


Figure 9.1.4 Control of Transciprtion CAP

Although the presence of lactose removes the repressor, the presence of glucose lowers the level of cAMP in the cell and thus removes CAP.Without CAP, binding of RNA polymerase is inhibited even though there is no repressor to interfere with it if it could bind. The molecular basis for its choices is shown in the above figure.

CAP consists of two identical polypeptides (hence it is a homodimer). Toward the C-terminal, each has two regions of alpha helix with a sharp bend between them. The longer of these is called the **recognition helix** because it is responsible for recognizing and binding to a particular sequence of bases in DNA.

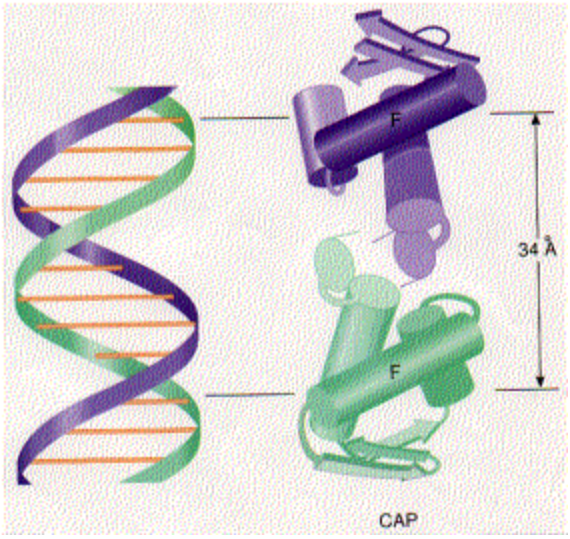


Figure 9.1.5 Model of CAP

The above figure shows a model of CAP. The two monomers are identical. Each monomer recognizes a sequence of nucleotides in DNA by means of the region of alpha helix labeled **F**. Note that the two recognition helices are spaced 34Å apart, which is the distance that it takes the DNA molecule (on the left) to make precisely one complete turn.

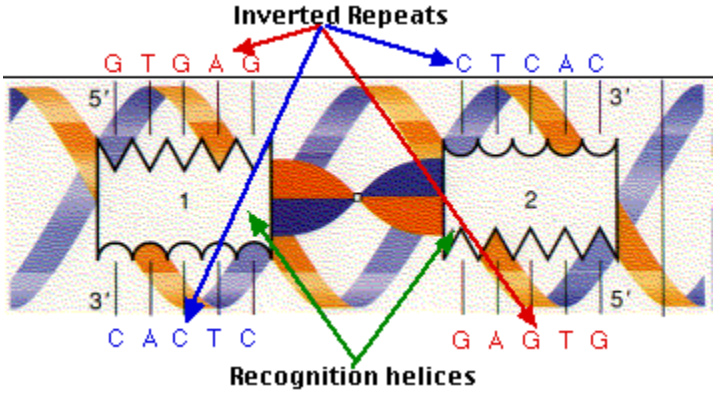


Figure 9.1.6 Recognition Helix

The recognition helices of each polypeptide of CAP are, of course, identical. But their orientation in the dimer is such that the sequence of bases they recognize must run in the opposite direction for each recognition helix to bind properly. This arrangement of two identical sequences of base pairs running in opposite directions is called an **inverted repeat**.
The strategy illustrated by CAP and its binding site has turned out to be used widely. As more and more DNA-regulating proteins have been discovered, many turn out to share the traits we find in CAP:

- They usually contain two subunits. Therefore, they are **dimers**.
- They recognize and bind to DNA sequences with **inverted repeats**.
- In bacteria, recognition and binding to a particular sequence of DNA is accomplished by a segment of alpha helix. Hence these proteins are often described as **helix-turn-helix** proteins. The Trp repressor shown above is a member of this group.

Riboswitches

Protein repressors and corepressors are not the only way in which bacteria control gene transcription. It turns out that the regulation of the level of certain metabolites can also be controlled by riboswitches. A riboswitch is section of the 5'-untranslated region (5'-UTR) in a molecule of messenger RNA (mRNA) which has a specific binding site for the metabolite (or a close relative). Some of the metabolites that bind to riboswitches include:

- the purines adenine and guanine

- the amino acids glycine and lysine
- flavin mononucleotide (the prosthetic group of NADH dehydrogenase)
- S-adenosyl methionine that donates methyl groups to many molecules, including DNA and the cap at the 5' end of messenger RNA
- tRNAs. When these are bound to their amino acid (aminoacyl-tRNA), they bind to the riboswitch in the mRNA that encodes the enzyme (an aminoacyl-tRNA synthetase) responsible for loading the amino acid onto the tRNA. This causes transcription of the mRNA to terminate prematurely. tRNAs with no amino acid attached also bind to the riboswitch but in such a way that transcription of the mRNA continues. Its translation (in bacteria, translation begins while transcription is still going on) produces the aminoacyl-tRNA synthetase used to load the amino acid onto the tRNA. Thus these riboswitches regulate the level of aminoacyl-tRNAs producing more when needed, less when not (a kind of feedback inhibition.)

In each case, the riboswitch regulates **transcription** of genes involved in the metabolism of that molecule. The metabolite binds to the growing mRNA and induces an allosteric change that for some genes causes further synthesis of the mRNA to terminate before forming a functional product and for other genes, enhances completion of synthesis of the mRNA. In both cases, one result is to control the level of that metabolite.

Some riboswitches control mRNA **translation** rather than its transcription. It has been suggested that these regulatory mechanisms, which **do not involve any protein**, are a relict from an "RNA world".

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9.2: The Tryptophan Repressor

In *E. coli*, the synthesis of the amino acid tryptophan from precursors available to the cell requires 5 enzymes. The genes encoding these are clustered together in a single **operon** with its own **promoter** and **operator**. When tryptophan is **available** to the cell, its presence **shuts down** the operon.

Mechanism

- One molecule of tryptophan binds to a site on each monomer of the Trp repressor.
- The **Trp repressor**, a homodimer of two of these complexes, binds to the operator of the **Trp operon**.
- This shuts down transcription of the 5 genes of the operon so the enzymes used in Trp synthesis are not synthesized.

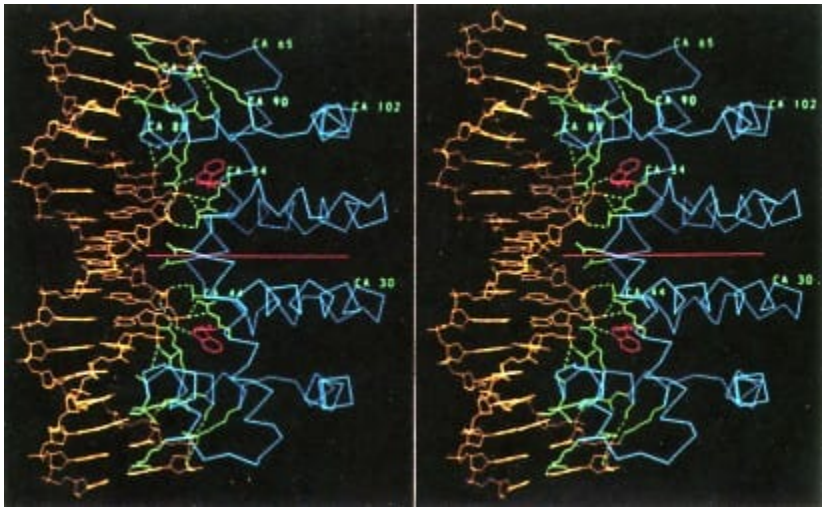


Figure 9.2.1: The Tryptophan Repressor courtesy of P. B. Sigler

This stereoscopic view () shows the tryptophan repressor (right side of each panel) bound to its operator DNA (left side). The two identical polypeptides of the repressor are shown on either side of the horizontal red line. The two tryptophan molecules are shown as red rings. Look also for the stretches of alpha helix in each monomer. You may find it easier to fuse the two images into a 3D view by holding a sheet of 8.5 x 11" (22 x 28 cm) paper vertically between your nose and the dividing line between the two images on the screen so that your left eye sees only the left image, your right eye only the right.

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9.3: Regulation of Gene Expression in Eukaryotes

The latest estimates are that a human cell, a eukaryotic cell, contains some 21,000 genes. Some of these are expressed in all cells all the time. These so-called **housekeeping genes** are responsible for the routine metabolic functions (e.g. respiration) common to all cells. Some are expressed as a cell enters a particular pathway of differentiation. Some are expressed all the time in only those cells that have differentiated in a particular way. For example, a plasma cell expresses continuously the genes for the antibody it synthesizes. Some are expressed only as conditions around and in the cell change. For example, the arrival of a hormone may turn on (or off) certain genes in that cell.

How is gene expression regulated? There are several methods used by eukaryotes.

- Altering the **rate of transcription** of the gene. This is the most important and widely-used strategy.
- However, eukaryotes supplement transcriptional regulation with several other methods:
  - Altering the rate at which RNA transcripts are processed while still within the nucleus.
  - Altering the stability of messenger RNA (mRNA) molecules; that is, the rate at which they are degraded.
  - Altering the efficiency with which ribosomes translate the mRNA into a polypeptide.

Protein-coding genes have

- **exons** whose sequence encodes the polypeptide
- **introns** that will be removed from the mRNA before it is translated
- **a transcription start site**
- **promoters**
  - a basal or **core promoter** located within about 40 base pairs (bp) of the start site
  - **"upstream" promoters**, which may extend over as many as 200 bp farther upstream
- **enhancers**
- **silencers**

Adjacent genes are often separated by an **insulator** which helps them avoid cross-talk between each other's promoters and enhancers (and/or silencers).

Transcription start site

This is where a molecule of **RNA polymerase II** (**pol II**, also known as **RNAP II**) binds. Pol II is a complex of 12 different proteins (shown in the figure in yellow with small colored circles superimposed on it). The start site is where transcription of the gene into RNA begins.

The core promoter

All eukaryotic genes contain a core promoter. One common example is a sequence of bases (e.g., TATAAAAAA) called the **TATA box**. It is bound by a large complex of some 50 different proteins, including

- Transcription Factor IID (**TFIID**) which is a complex of
  - **TATA-binding protein** (**TBP**), which recognizes and binds to the TATA box
  - 13 other protein factors which bind to TBP, each other, and (some of them) to the DNA.
- **Transcription Factor IIB** (**TFIIB**) which binds both the DNA and pol II.

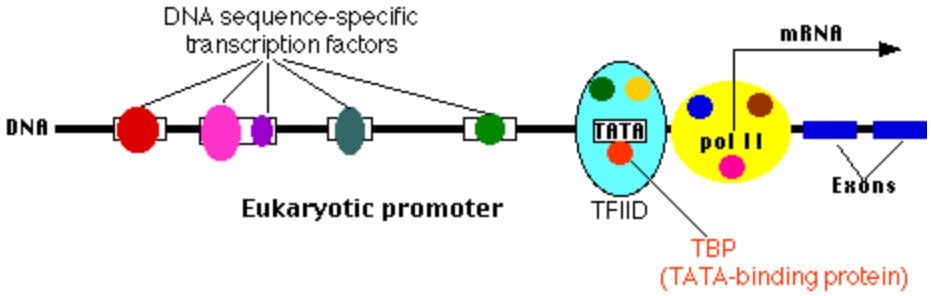


Figure 9.3.1: Eukaryotic Promoter

Many different genes and many different types of cells share the same transcription factors — not only those that bind at the core promoter but even some of those that bind upstream. What turns on a particular gene in a particular cell is probably the unique **combination** of promoter sites and the transcription factors that are chosen.

An Analogy

The rows of lock boxes in a bank provide a useful analogy. To open any particular box in the room requires two keys:

- Your key, whose pattern of notches fits only the lock of the box assigned to you (= the upstream promoter), but which cannot unlock the box without
- A key carried by a bank employee that can activate the unlocking mechanism of any box (= the core promoter) but cannot by itself open any box.

Hormones Effect

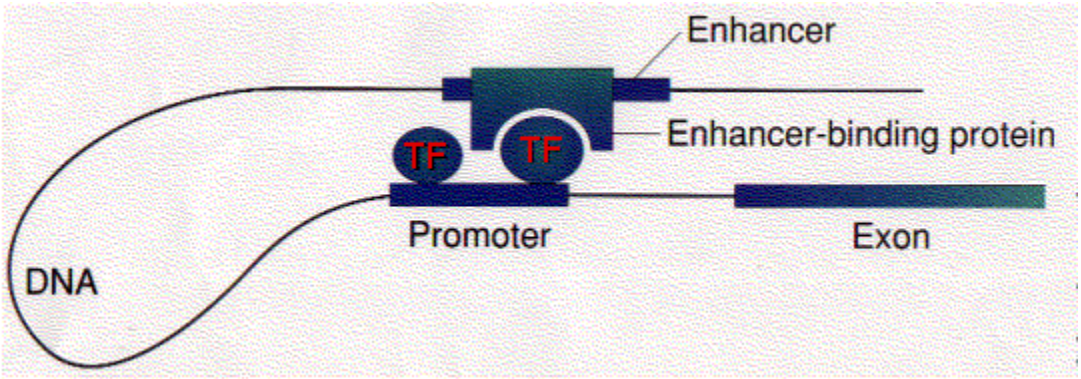


Figure 9.3.2: Enhancer

These loops are stabilized by a protein designated **CTCF** ("CCCTC binding factor"; named for the nucleotide sequence to which it binds). The CTCF at one site on the DNA forms a dimer with the CTCF at another site on the DNA binding the two regions together. CTCF has 11 zinc fingers. They can also be stabilized by **cohesin** — the same protein complex that holds sister chromatids together during mitosis and meiosis.

Michael R. Botchan and his colleagues have produced visual evidence of this model of enhancer action. They created an artificial DNA molecule with

- several (4) **promoter sites for Sp1** about 300 bases from one end. Sp1 is a zinc-finger transcription factor that binds to the sequence 5' GGGCGG 3' found in the promoters of many genes, especially "housekeeping" genes.
- several (5) **enhancer sites** about 800 bases from the other end. These are bound by an enhancer-binding protein designated **E2**.
- 1860 base pairs of DNA between the two.

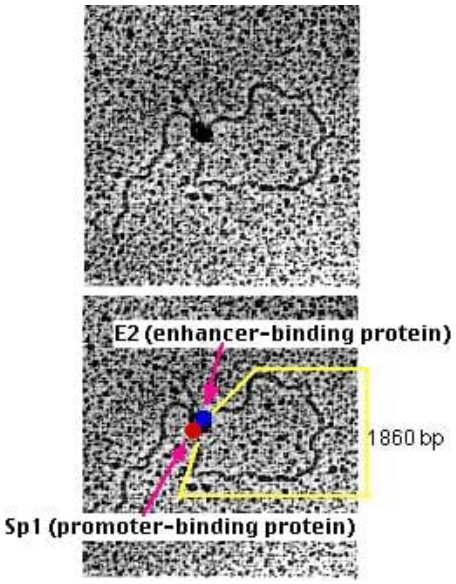


Figure 9.3.3: Evidence of Enhancer Action courtesy Michael R. Botchan

Significance of "Looping"

The looping of chromosomes that brings enhancers close to promoters (and promoters close to other promoters) seems to be a mechanism to ensure the expression (or inhibition) of groups of genes that must perform together. The response of a cell to the arrival of a signal (e.g., a hormone) may involve turning on (or off) hundreds of different genes whose products must be produced in a coordinated way for the cell to respond appropriately. The dynamic movement of portions of the chromosome carrying the appropriate gene loci into a "transcription factory" may be a mechanism to accomplish this. If so, we are seeing the eukaryotic equivalent of the coordinated gene expression provided by **operons** in bacteria.

Silencers

Silencers are control regions of DNA that, like enhancers, may be located thousands of base pairs away from the gene they control. However, when transcription factors bind to them, expression of the gene they control is repressed.

Insulators

As you can see above, enhancers can turn on promoters of genes located thousands of base pairs away. Insulators prevent an enhancer from inappropriately binding to and activating the promoter of some other gene in the same region of the chromosome..

Insulators are

- stretches of DNA (as few as 42 base pairs may do the trick)
- located between the
  - enhancer(s) and promoter(s) or
  - silencer(s) and promoter(s)

  of **adjacent** genes or clusters of adjacent genes.

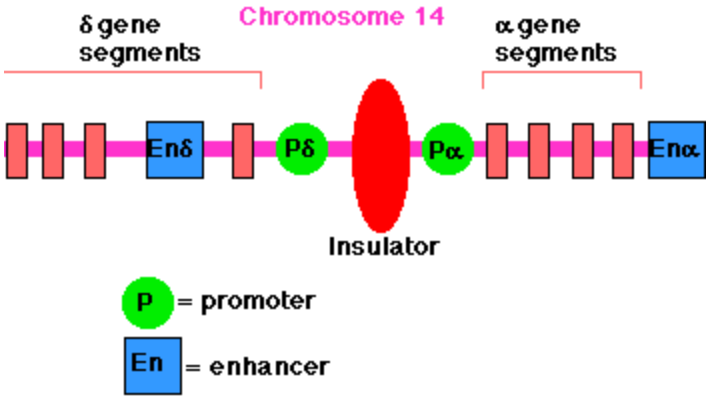


Figure 9.3.4 Insulator

The enhancer for the promoter of the gene for the delta chain of the **gamma/delta** T-cell receptor for antigen (**TCR**) is located close to the promoter for the alpha chain of the **alpha/beta** TCR (on chromosome 14 in humans). A T cell must choose between one or the other. There is an insulator between the alpha gene promoter and the delta gene promoter that ensures that activation of one does not spread over to the other.

All insulators discovered so far in vertebrates work only when bound by the **CTCF** protein. **Another example:** In mammals (mice, humans, pigs), only the allele for **insulin-like growth factor-2** (***IGF2***) inherited from one's father is active; that inherited from the mother is not — a phenomenon called **imprinting**.

The mechanism: the mother's allele has an insulator between the *IGF2* promoter and enhancer. So does the father's allele, but in his case, the insulator has been methylated. CTCF can no longer bind to the insulator, and so the enhancer is now free to turn on the father's *IGF2* promoter.

Many of the commercially-important varieties of pigs have been bred to contain a gene that increases the ratio of skeletal muscle to fat. This gene has been sequenced and turns out to be an allele of *IGF2*, which contains a single point mutation in one of its **introns**. Pigs with this mutation produce higher levels of *IGF2* mRNA in their skeletal muscles (but not in their liver). This tells us that:

- Mutations need not be in the protein-coding portion of a gene in order to affect the phenotype.
- Mutations in non-coding portions of a gene can affect how that gene is **regulated** (here, a change in muscle but not in liver).

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9.4: Steroid Response Elements

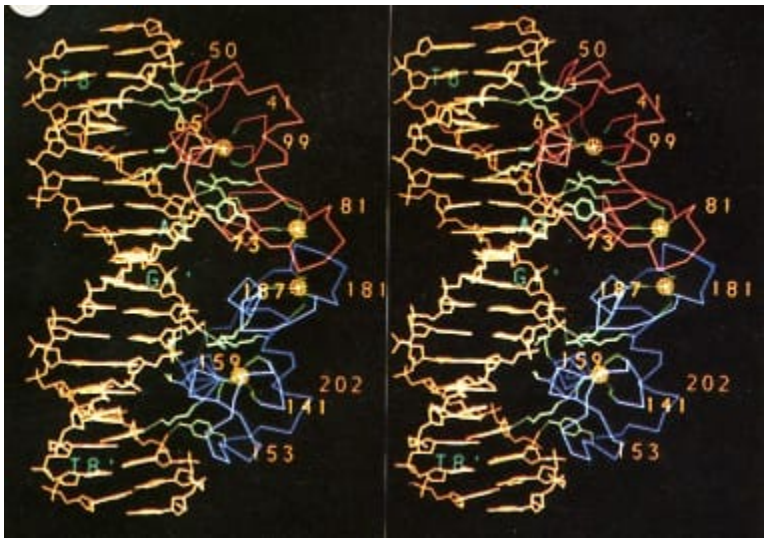


Figure 9.4.1: Stereoscopic view of the glucocorticoid response element courtesy of P. B. Sigler. This image shows a stereoscopic view of the **glucocorticoid response element** (DNA, the double helix shown in yellow at the left of each panel) with the **glucocorticoid receptor** (a protein homodimer, right portion of each panel) bound to it.

The DNA sequence of the glucocorticoid response element is

5' AGAACAnnnTGTTCT 3'
3' TCTTGTnnnACAAGA 5'

where **n** represents any nucleotide. (Note the inverted repeats.) The glucocorticoid receptor, like all steroid hormone receptors, is a **zinc-finger transcription factor**; the zinc atoms are the four yellow spheres. Each is attached to four cysteines.

For a steroid hormone to regulate (turn on or off) gene transcription, its receptor must:

- bind to the hormone (cortisol in the case of the glucocorticoid receptor)
- bind to a second copy of itself to form a **homodimer**
- be in the nucleus, moving from the cytosol if necessary
- bind to its response element
- bind to other protein cofactors

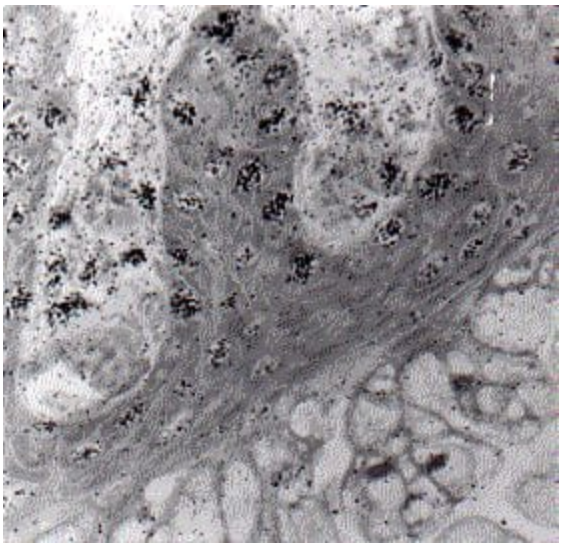


Figure 9.4.2: Autoradiograph of Endometrial cell courtesy of Madhabananda Sar and Walter E. Stumpf

This autoradiograph shows the endometrial cells from the uterus of a guinea pig 15 minutes after an injection of radioactive **progesterone**. The radioactivity has concentrated within the nuclei of the endometrial cells as shown by the dark grains superimposed on the images of the nuclei. The same effect is seen when radioactive **estrogens** are administered.

The cells of the endometrium are target cells for both progesterone and estrogens, preparing the uterus for possible pregnancy. Nontarget cells (e.g. liver cells or lymphocytes) show no accumulation of female sex hormones. Although their DNA contains the response elements, their cells do not have the protein receptors needed.

The Nuclear Receptor Superfamily

The zinc-finger proteins that serve as receptors for glucocorticoids and progesterone are members of a large family of similar proteins that serve as receptors for a variety of small, hydrophobic molecules. These include:

- other steroid hormones like the mineralocorticoid aldosterone and estrogens
- the thyroid hormone, T3
- calcitriol, the active form of vitamin D
- retinoids: vitamin A (**retinol**) and its relatives
- retinal
- retinoic acid (tretinoin — also available as the drug Retin-A®); and its isomer
- isotretinoin (sold as Accutane® for the treatment of acne).
- bile acids
- fatty acids. These bind members of the superfamily called peroxisome-proliferator-activated receptors (**PPARs**). They got their name from their initial discovery as the receptors for drugs that increase the number and size of peroxisomes in cells.
- In every case, the receptors consists of at least three functional modules or **domains**. From N-terminal to C-terminal, these are:
  - a domain needed for the receptor to activate the promoters of the genes being controlled
  - the zinc-finger domain needed for DNA binding (to the response element)
  - the domain responsible for binding the particular hormone as well as the second unit of the dimer

Contibutors

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9.5: Epigenetics

Epigenetics can be defined as a change in **phenotype** that is heritable but does **not** involve a change in the nucleotide sequence in DNA; that is, a change in **genotype**. This definition is very broad encompassing a variety of phenomena.

Epigenetic changes during cellular differentiation

For example, a change in phenotype of a single cell that is then passed on to its descendants qualifies as an epigenetic phenomenon. Thus it includes the various pathways of **differentiation** that are taken by cells during the embryonic development of an organism. Examples:

- X-inactivation — where one of the two X chromosomes in female mammals is inactivated in each cell early in development and that same chromosome remains inactivated in all the descendants of that cell.
- **Imprinting** — where whether a gene in a cell lineage is expressed or not depends on which parent contributed the gene.

The great stumbling block in converting differentiated cells into induced pluripotent stem cells (iPSCs) was to find ways of reversing the epigenetic changes in the differentiated cell (e.g., a skin cell) to unlock its full developmental potential. Stable changes in gene expression are brought about in two main ways:

- DNA methylation — where its cytosines are methylated. This usually represses the activity of that DNA.
- Histone modifications — where methyl, acetyl, and other groups are added to the histones in chromatin. Prominent examples:
  - adding methyl groups to the #4 lysine in histone H3 ("H3K4me"). This is associated with **active** genes in that region of the chromatin.
  - adding methyl groups to the #27 lysine in histone H3 ("HeK27me"). This is associated with gene **silencing**.

Some definitions:

- epigenetic "**writers**": enzymes that add chemical groups to histones or DNA.
- epigenetic "**erasers**": enzymes that remove these groups.
- epigenetic "**readers**": proteins that recognize specific epigenetic modifications of histones or DNA producing a change in gene expression, e.g., increasing (or decreasing) gene transcription.

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9.6: Visualization of Transcription and Translation in Bacteria

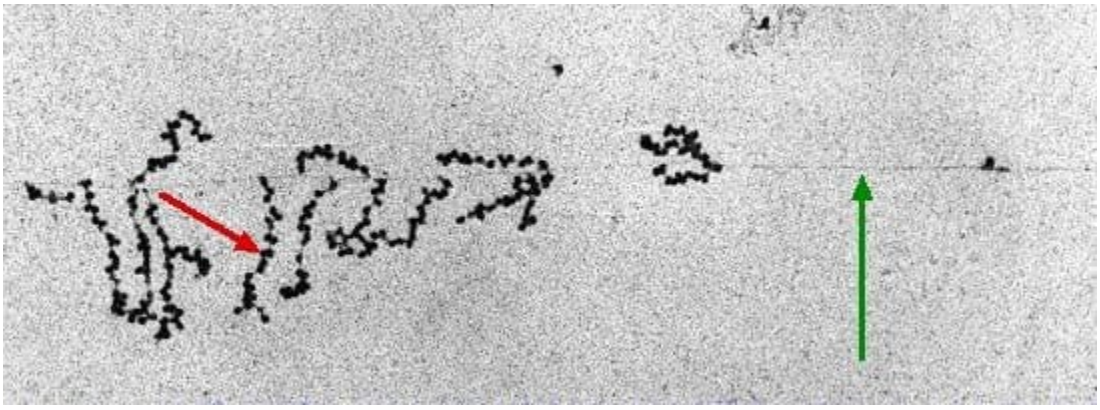


Figure 9.6.1: Transcition and Translationn of E. coli genes

The above figure is an Electron micrograph courtesy of O. L. Miller, Jr., B. A. Hamkalo, and C. A. Thomas, Jr. It shows simultaneous **transcription** and **translation** of *E. coli* genes. The long fiber running from top to bottom (green arrow) is a segment of the *E. coli* chromosome. Extending from it are **polysomes** (red arrow), the size of which generally increases from top to bottom. Each polysome consists of a backbone of **messenger RNA** (**mRNA**) to which the **ribosomes** are attached.

Each polysome is attached to the DNA fiber by a complex of proteins that includes a molecule of **RNA polymerase**. Thus the DNA is **transcribed by RNA polymerase** molecules moving from top to bottom, and the growing mRNA molecules are **translated by ribosomes** moving in a proximal -> distal direction. IIn E. coli, then, and probably in all bacteria, the **transcription** of DNA into mRNA and the **translation** of mRNA into polypeptides (not visible here) are closely coordinated in both time and space.

In eukaryotes, in contrast, while all transcription takes place in the nucleus, most (but not all) translation of mRNA occurs later in the cytosol.

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9.7: Footprinting

Footprinting is a method for determining the exact DNA sequence to which a particular DNA-binding protein binds. Examples:

- hormone-receptor complexes that bind to their hormone response elements
- transcription factors that bind eukaryotic operators, enhancers, and silencers
- the **lac repressor** that shuts down the *lac* operon in *E. coli*

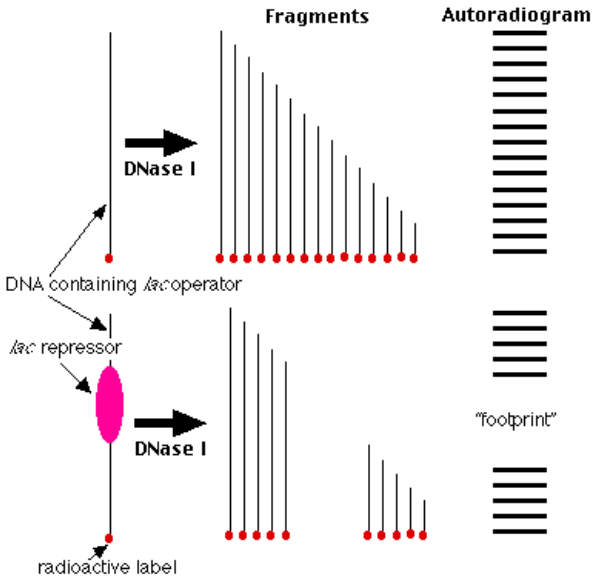


Figure 9.7.1 Footprinting

- Clone a piece of DNA that contains the operator site to which the repressor binds.
- Label one end of the DNA molecules with a radioactive molecule, e.g. radioactive ATP.
- Digest the DNA with **DNase I**.
  - DNase I cuts DNA molecules randomly (in contrast to restriction enzymes that cut where they find a particular sequence)
  - Choose such gentle conditions that most molecules will be cut only once.
- The result will be a mixture of radioactive fragments of varying length, with the smallest increment in length represented by a single nucleotide.
- Separate the fragments by electrophoresis.
- Binding of the *lac* repressor to the sequence of 24 base pairs in the operator prevents DNase I from attacking that region of the molecule.
- When the fragments are separated by electrophoresis, those representing the lengths covered by the repressor will be missing from the autoradiogram.
- The resulting gap is the "footprint".
- The same sample of DNA (unprotected by the repressor) is subjected to normal DNA sequencing and the resulting ladder aligned with the footprint autoradiogram.
- The exact sequence of bases in the *lac* operator can then be read directly because they represent the rungs of the ladder missing in the footprint.

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9.8: Chromatin Immunoprecipitation

Many DNA-binding proteins, such as transcription factors, bind to specific sequences of nucleotides in, for example, promoters and enhancers of genes. Some examples:

- a generalized eukaryotic promoter
- multiple transcription factors bound to the Drosophila *eve* promoter
- the glucocorticoid receptor (protein) bound to its response element (DNA)
- the tryptophan repressor bound to its operator

The binding of protein to DNA is done by noncovalent forces and is easily reversible. In fact, as conditions in a cell change, there is a dynamic coming and going of DNA-binding proteins throughout the genome. The identification of a specific site in DNA bound by a particular protein at a particular time can be discovered by the technique of **chromatin immunoprecipitation** (ChIP).

The Procedure

1. Harvest your cell population at the desired time. Some examples:
    - yeast cells growing on a particular nutrient;
    - HeLa cells exposed to a particular cytokine;
    - salivary gland cells of Drosophila at the time of pupation.
2. Treat the cells with formaldehyde (HCHO) which creates covalent bonds between the proteins and nucleotides to which they have been bound noncovalently.
3. Break open the cells releasing their contents.
4. Use ultrasound to break the DNA into fragments averaging about 500 bp long.
5. Add an antibody that specifically binds to the protein you are interested in.
6. Add beads coated with Protein A or Protein S — both proteins that bind to any antibody.
7. Centrifuge down the complexes of bead—antibody—target protein—DNA.
8. Heat the complexes to break the covalent crosslinks between the target protein and the DNA.
9. Digest the protein with a protease leaving purified DNA fragments.
10. Perform PCR on the fragments.
11. Use any of several methods to identify the amplified DNA, for example,
    - Southern blotting
    - DNA chip analysis ("ChIP on chip")
    - clone and sequence ("ChIP-Seq")

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9.9: Isolating Transcription Factors

Transcription factors are extraordinarily diverse, and any one factor represents only a tiny fraction of the protein molecules present in the cell. This page describes how one can isolate and purify such rare molecules.

Example: Isolating the *lac* Repressor

An ***E. coli*** cell contains only 10-20 copies of the *lac* repressor. This represents a ratio of only 1 molecule in 50,000 protein molecules in the cell. However, the specificity of the *lac* repressor for the DNA sequence of the operator provides a mechanism for fishing it out of the mixture.

Affinity Chromatography

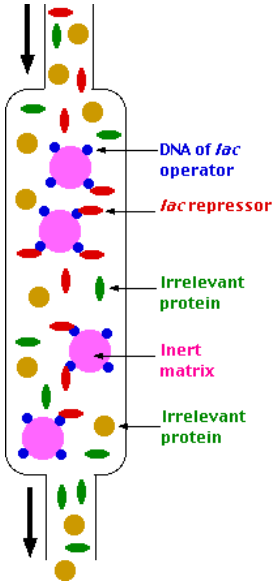


Figure 9.9.1 Affinity Chromatography

The following procedure is carried out for an affinity chromatograph:

- Learn the sequence to which the repressor binds (by **footprinting**).
- Synthesize a segment of DNA containing the sequence.
- Attach this artificial molecule to beads of an inert, solid medium (the matrix).
- Pour an extract of ***E. coli*** cells over the beads.
- Only molecules specific for the DNA sequence — in this case, molecules of the *lac* repressor — will bind to the beads.
- After irrelevant protein molecules have passed through the column, wash the beads with a buffer that will release the *lac* repressor molecules so they can be studied.

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9.10: Palindromes

A palindrome is a sequence of letters and/or words, that reads the same forwards and backwards. "**able was I ere I saw elba**" is a palindrome. Palindromes also occur in a DNA. There are two types.

Palindromes that occur on opposite strands of the same section of DNA helix

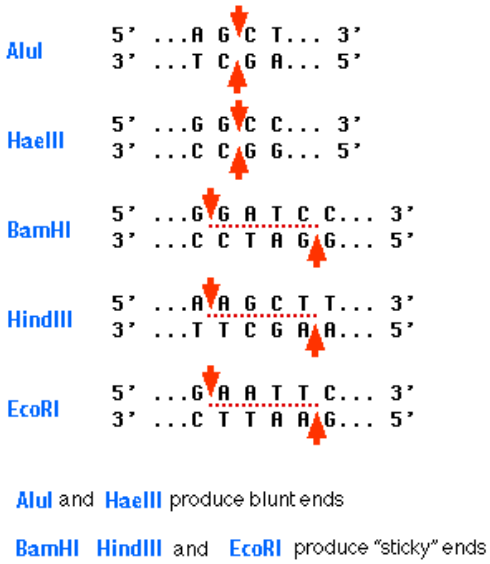


Figure 9.10.1 Restriction Enzymes Palindrome

5' GGCC 3'
3' CCGG 5'

This type of palindrome serves as the target for most restriction enzymes. The graphic shows the palindromic sequences "seen" by five restriction enzymes (named in blue) commonly used in recombinant DNA work.

Inverted Repeats

In these cases, two different segments of the double helix read the same but in opposite directions.

5' AGAACAnnnTGTTCT 3'
3' TCTTGTnnnACAAGA 5'

Inverted repeats are commonly found in

- The DNA to which transcription factors bind.

  The DNA sequence shown above is that of the **glucocorticoid response element** where **n** represents any nucleotide. Transcription factors are often dimers of identical proteins **homodimers** so it is not surprising that each member of the pair needs to "see" the same DNA sequence in the same orientation.

- The DNA of many **transposons** is flanked by inverted repeats such as this one:

  - **5' GGCCAGTCACAATGG..~400 nt..CCATTGTGACTGGCC 3'**
    3' CCGGTCAGTGTTACC..~400 nt..GGTAACACTGACCGG 5'

- Inverted repeats at either end of retroviral gene sequences aid in inserting the DNA copy into the DNA of the host.
- Duplicated Genes.

  The human Y chromosome contains 7 sets of genes — each set containing from 2 to 6 nearly-identical genes — oriented back-to-back or head-to-head; that is, they are inverted repeats like the portion shown here. (The dashes represent the thousands of base pairs that separate adjacent palindromes.)

  CTCCCACAACCCATGGGATTTGTG... 3'

  GAGGGTGTTGGGTACCCTAAACAC... 5'

  This orientation and redundancy may help ensure that a deleterious mutation in one copy of the set can be repaired using the information in another copy of that set. All that is needed is to form a loop so that the two sequences line up side-by-side. Repairs can then be made (probably by the mechanism of homologous recombination). Here, for example, the single difference in the sequences can be eliminated (red for blue or vice versa).

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9.11: Cell-specific gene expression

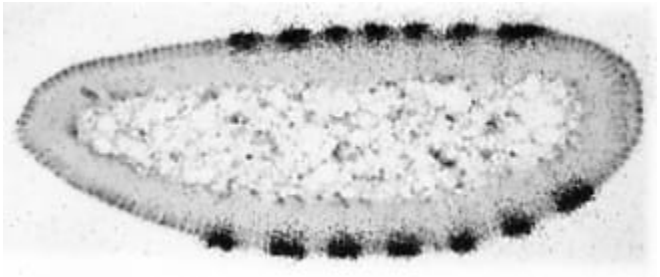


Figure 9.11.2 Even-skipped gene courtesy of Peter A. Lawrence and Blackwell Scientific Publications

An example: Make Drosophila transgenic for recombinant DNA containing:

- the ***Z* gene** for **beta-galactosidase**
- ***even-skipped*** (***eve***), (another homeobox gene).

Any cell with the transcription factors for turning on the *even-skipped* promoter will begin to make beta-galactosidase. Given the proper substrate, the enzyme produces a colored product.

The above photomicrograph shows 7 bands of this colored product identifying the cells that were expressing the *even-skipped* gene. This event was "reported" by the *lacZ* gene. The 7 dark stripes reveal regions that alternate with the 7 bands formed by the cells expressing *fushi-tarazu*.

Green fluorescent protein (GFP)

In nature, green fluorescent protein (GFP) is produced by, **Aequorea victoria**, the Pacific Northwest jellyfish. The protein has become of great interest to cell and molecular biologists because it can reveal gene expression in living cells.

This is done by fusing the gene for GFP to the gene whose expression you are interested in. When that gene is turned on in a cell, not only is its protein synthesized, but GFP is synthesized as well. Illuminating the cells with near-ultraviolet light causes them to fluoresce a bright green. In this way, the experimenter can see when and where the gene is expressed in the living organism.

DNA Chips

All the methods described so far are limited to monitoring the expression of one or, at most, a few genes. But as conditions change in a cell, the transcription and translation of literally hundreds of genes may be altered.

Thanks to the marriage of

- semiconductor chip technology
- automated synthesis of oligodeoxynucleotides
- automated fluorescence scanners
- computer software,

it is now possible to monitor the activity of literally thousands of genes in one kind of cell. For examples:

- mammalian cells when they are transferred from a "minimal" culture medium to one enriched in growth factors;
- the skeletal muscles of mice as they age.

The Chip

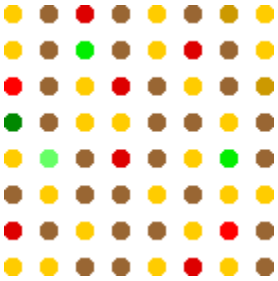


Figure 9.11.3 DNA chip

- Examine published gene sequences.
- For each gene, pick out ~20 different stretches of ~25 nucleotides that seem characteristic of that gene.
- Synthesize oligodeoxynucleotides corresponding to these.
- Also synthesize oligodeoxynucleotides for each of the above that have one nucleotide altered (usually near the middle). These will provide a control.
- Using robotic chip-making machines, spot these oligonucleotides individually in arrays, each spot receiving millions of copies that are fixed to the chip surface.

With the partial completion of the human genome project, three companies are now selling DNA chips containing from 36,000 to 50,000 pieces of DNA thought to represent different human genes.

The Assay

- Harvest your cells. Presumably they are expressing a characteristic **subset of their genes**; that is, transcribing them into messenger RNA (**mRNA**) molecules.
- Extract the RNA.
- Make complementary DNA (**cDNA**) by treating the RNA mixture with reverse transcriptase.
- Transcribe the cDNA back into now much-amplified RNA.
- Attach fluorescent tags to the RNA.
- Flood the chip with this mixture.
- RNAs finding their complementary sequences on the chip will bind to them. (They will bind less strongly to adjacent spots with the single-nucleotide change if the binding is truly specific.
- Illuminate the chip and automatically record the intensities of the color at each spot.
- Use a computer to analyze the pattern.

Results of monitoring genome-wide expression

- This work was reported by V. R. Iyer, et al in the 1 January 1999 issue of **Science**. It involved the monitoring the expression of **8613** different genes.
- Mice raised on a restricted diet did not show such dramatic shifts in gene expression as they aged. This fits well with data that mice on restricted diets age more slowly than those on rich diets.

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9.12: Imprinted Genes

Imprinted genes are genes whose expression is determined by the parent that contributed them. Imprinted genes violate the usual rule of inheritance that both alleles in a heterozygote are equally expressed.

Examples of the **usual rule**:

- If a child inherits the gene for **blood group A** from either parent and the gene for **group B** from the other parent, the child's blood group will be **AB**.
- If a child inherits the gene encoding **hemoglobin A** from either parent and the gene encoding **hemoglobin S** from the other parent, the child's red blood cells will contain roughly equal amounts of the two types of hemoglobin.

But there are a few exceptions to this rule. A small number of genes in mammals (~80 of them at a recent count) and in angiosperms have been found to be **imprinted**. Because most imprinted genes are repressed, either

- the **maternal** (inherited from the mother) allele is expressed exclusively because the **paternal** (inherited from the father) allele is imprinted or
- vice versa.

The process begins during gamete formation when

- in males certain genes are imprinted in developing sperm and
- in females, others are imprinted in the developing egg.

All the cells in a resulting child will have the same set of imprinted genes from both its father and its mother EXCEPT for those cells ("germplasm") that are destined to go on to make gametes. All imprints — both maternal and paternal — are erased in them.

Examples

IGF2

— the gene encoding the **insulin-like growth factor-2**

In humans (and other mammals like mice and pigs) the *IGF2* allele inherited from the father (paternal) is expressed; the allele inherited from the mother is not.

If both alleles should begin to be expressed in a cell, that cell may develop into a cancer.

IGF2r

— the gene encoding the cell **receptor for Igf-2**

In mice the *IGF2r* allele inherited from the mother is expressed; that from the father is not. Differential imprinting accounts for this, and the mechanism is described below.

XIST

— the gene encoding the RNA that converts one of the X chromosomes in a female cell into an inactive **Barr body**. This process is random in the cells of the female **fetus** and thus is NOT an example of imprinting. However, all the cells of her extraembryonic membranes (which form the amnion, placenta, and umbilical cord) have the father's X chromosome inactivated. Imprinting of the *XIST* locus accounts for this.

Mechanism of parental imprinting

The process of imprinting starts in the gametes where the allele destined to be inactive in the new embryo (either the father's or the mother's as the case may be) is "marked". The mark appears to be **methylation** of the DNA in the promoter(s) of the gene.

Methyl groups are added to cytosines (Cs) in the DNA. When this occurs at stretches of alternating Cs and Gs called **CpG sites** in a promoter, it prevents binding of transcription factors to the promoter thus shutting down expression of the gene.



Although methylation seems to be the imprinting **signal**, keeping the gene shut down may require the production of **RNA**.

Methylation — and thus inactivation — of the promoters of tumor suppressor genes is frequently found in cancer cells.

The *IGF2r* gene

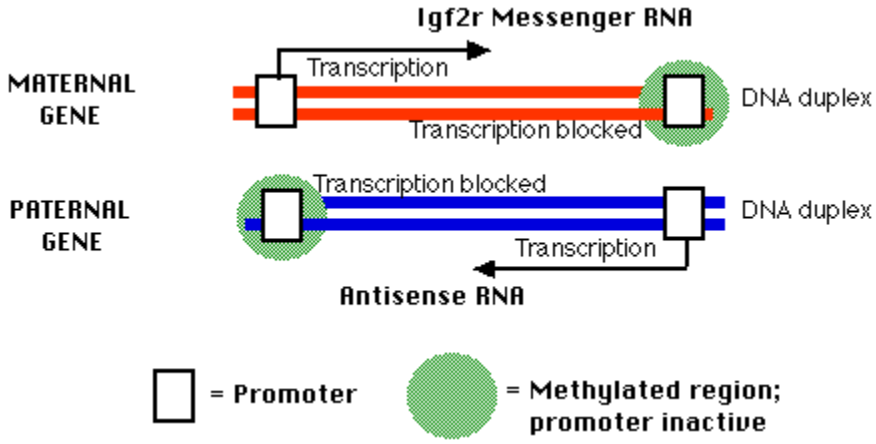


Figure 9.12.1 the IGF2r gene

A report in **Nature** (16 October 1997) by Wutz et al, reveals that:

In the mother's (**maternal**) copy of the gene,

- there is an upstream (left) promoter that is unmethylated and active
- binding of transcription factors to this upstream promoter enables transcription of the sense strand of the gene to produce **Igf2r messenger RNA**.
- There is also a downstream set of CpG sites that are methylated

In the father's (**paternal**) copy of the *IGF2r* gene (the imprinted version)

- the promoter for *IGF2r* transcription is methylated (and inactive),
- but the downstream promoter is unmethylated and active.
- Transcription of the antisense strand from the downstream promoter produces an **antisense RNA** (a long noncoding RNA) that participates in shutting his gene down.

XIST

The *XIST* locus on the X chromosome encodes a **long noncoding RNA** that shuts down all (or almost all) of the other genes on the chromosome, converting it into an inactive **Barr body**.

Is imprinting important?

Yes.

- Deliberate (in mice) or accidental (in humans) inheritance of two copies of a particular chromosome from one parent and none from the other parent is usually fatal (even though a complete genome is present).
- Inheritance of two copies of one of mother's genes and no copy of the father's (or vice versa) can produce serious developmental defects.
  - Failure to inherit several nonimprinted genes on the father's chromosome #15 causes a human congenital disorder called **Prader-Willi syndrome**.
  - Absence or mutation of a nonimprinted gene (*UBE3A*) on the mother's chromosome #15 causes **Angelman syndrome**.
- Failure of imprinting in somatic cells may lead to **cancer**.
  - The cancerous cells in some cases of a malignancy called **Wilms´ tumor** and many cases of colon cancer have both copies of the ***IGF2*** gene expressed (where only one, the father's, should be).
  - **Reduced methylation** — and hence increased expression — of **proto-oncogenes** can lead to cancer, while
  - **increased methylation** — and hence decreased expression — of **tumor suppressor genes** can also do so.

Imprinting and Parthenogenesis

Imprinting is the reason that parthenogenesis ("virgin birth") does not occur in mammals. Two complete female genomes cannot produce viable young because of the imprinted genes. For example, the embryo needs the father's *Igf2* gene because the mother's copy has been imprinted and is inactive.

- An insulator — with a bound protein designated CTCF ("CCCTC binding factor") (named for a nucleotide sequence found in all insulators) — prevents her *Igf2* gene from interacting with the enhancers needed to turn it ON.
- The father's copy of the gene can be turned on because methylation of his insulator prevents binding by CTCF so the enhancers can interact with the gene.

However, two healthy laboratory mice have been produced by parthenogenesis; that is, containing two female (haploid) genomes. (See Kono, T. *et al*., **Nature**, 22 April 2004.)

This was done by fusing two oocytes (thus each cell haploid):

- a normal oocyte with its imprinted (inactivated) *Igf2* gene
- an immature oocyte
  - harvested before imprinting occurs and
  - containing a deletion of the insulator that blocks enhancer activation of the *Igf2* gene. Thus the *Igf2* gene from this oocyte could be expressed in the developing embryo.

Out of several hundred attempts, two resulting blastocysts not only implanted successfully in a surrogate mother but went on to be born normally. One even grew up and had babies of her own.

Imprinting in Plants

Some genes in the endosperm of angiosperms are imprinted by the addition of methyl groups. For some, both maternal copies (endosperm is **3n**) are expressed (demethylated) while the male allele remains shut down. For other genes, it is the female alleles that are imprinted and thus not expressed while the male allele is functional.

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9.13: Ribozymes

Until about 20 years ago, all known enzymes were proteins. But then it was discovered that some RNA molecules can act as enzymes; that is, catalyze covalent changes in the structure of substrates (most of which are also RNA molecules). Catalytic RNA molecules are called ribozymes. Most classes of RNA, including transfer RNA (**tRNA**), ribosomal RNA (**rRNA**), and messenger RNA (**mRNA**) are transcribed as precursors that are larger than the final product. These precursors often contain "head" (5') and "tail" (3') sequences and intron sequences that must be removed to make the final product. Some of the processing steps employ other RNA molecules (always associated with proteins).

Ribonuclease P

Almost all living things synthesize an enzyme — called Ribonuclease P (RNase P) — that cleaves the head (5') end of the precursors of transfer RNA (tRNA) molecules. In bacteria, ribonuclease P is a heterodimer containing a molecule of RNA and one of protein. Separated from each other, the RNA retains its ability to catalyze the cleavage step (although less efficiently than the intact dimer), but the protein alone cannot do the job.

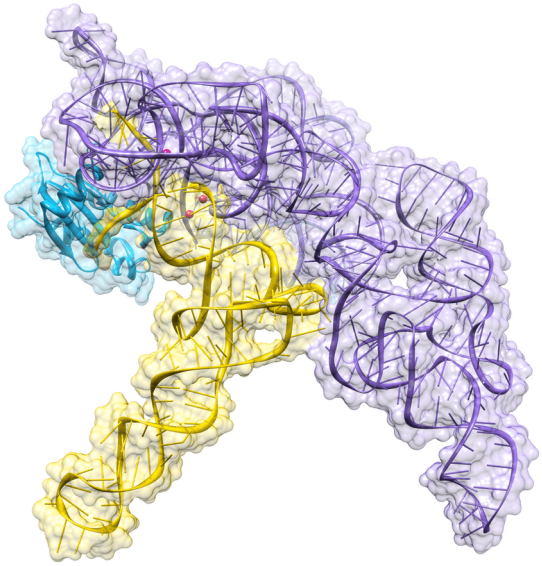


Figure 9.13.1: Crystal structure of a bacterial ribonuclease P holoenzyme in complex with tRNA (yellow), showing metal ions involved in catalysis (pink spheres), PDB: 3Q1R. (CC BY SA 3.0; RNAMacGyver).

Group I Introns

Some **ribosomal RNA** (**rRNA**) genes, including those in the mitochondrial genome of certain fungi (e.g., yeast), in some chloroplast genomes and in the nuclear genome of some "lower" eukaryotes (e.g., the ciliated protozoan *Tetrahymena thermophila* and plasmodial slime mold*Physarum polycephalum)* contain introns that must be spliced out to make the final product.

The splicing reaction is self-contained; that is, the intron — with the help of associated proteins — splices itself out of the precursor RNA. Once excision of the intron and splicing of the adjacent exons are completed, the story is over. In other words, although the action is catalyzed by the RNA, only a single molecule of substrate is involved (unlike protein enzymes that repeatedly catalyze a reaction).

However, synthetic versions of Group I introns made in the laboratory can — *in vitro* — act repeatedly; that is, like true enzymes. The DNA of some Group I introns includes an **open reading frame** (ORF) that encodes a transposase-like protein that can make a copy of the intron and insert it elsewhere in the genome. All the Group I introns share a characteristic secondary structure and mode of action that distinguishes them from the next group.

Group II Introns

Some **messenger RNA** (**mRNA**) genes in the mitochondrial genome of yeast and other fungi (encoding the proteins **cytochrome b** and subunits of **cytochrome c oxidase**) and in some chloroplast genomes also contain **self-splicing** introns. Because their secondary structure and the details of the splicing reaction differ from the rRNA introns discussed above, these are called Group II introns. The DNA of some Group II introns also includes an open reading frame (ORF) that encodes a transposase-like protein that can make a copy of the intron and insert it elsewhere in the genome.

Spliceosomes

Spliceosomes remove introns and splice the exons of most **nuclear genes**. They are composed of 5 kinds of small nuclear RNA (**snRNA**) molecules and over 100 different protein molecules. It is the RNA — not the protein — that catalyzes the splicing reactions. The molecular details of the reactions are similar to those of Group II introns, and this has led to speculation that this splicing machinery evolved from them.

Viroids

Viroids are DNA molecules that infect plant cells as conventional viruses do, but are far smaller (one has only 246 nucleotides). They are naked; that is, they are not encased in a capsi like viruses. Some viroidlike molecules get into the cell as passengers inside a conventional plant virus. These are called **virusoids** or **viroidlike satellite RNAs**.

In both cases, the molecules consists of single-stranded RNA whose ends are covalently bonded to form a circle. There are several regions where base-pairing occurs across adjacent portions of the molecule. New viroids and virusoids are synthesized by the host cell as long precursors in which the viroid structure is tandemly repeated. These repeats must be cut out and ligated to form the final product. Most virusoids and at least one viroid are **self-splicing**; that is, they can cut themselves out of the precursor and ligate their ends without the aid of any host enzymes. Thus they represent another class of **ribozyme**.

Both viroids and virusoids are responsible for a number of serious diseases of economically important plants, e.g. the coconut palm and chrysanthemums. (The problem is so severe with chrysanthemums that all growers in the U.S. now secure their stock from a few companies that raise the plants in "clean" rooms using stringent precautions to prevent infection by the viroid.)

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UNIT 10: MUTATION

10.1: Mutations - Causes and Significance
10.2: Testing for Mutagenic Chemicals in Bacteria and Mice
10.3: Radiation and its effect on DNA
10.4: Transposons - "jumping genes"

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10.1: Mutations - Causes and Significance

In the living cell, DNA undergoes frequent chemical change, especially when it is being replicated (in S phase of the eukaryotic cell cycle). Most of these changes are quickly repaired. Those that are not result in a mutation. Thus, **mutation is a failure of DNA repair**.

Single-base substitutions

A single base, say an A, becomes replaced by another. Single base substitutions are also called **point mutations**. (If one purine [A or G] or pyrimidine [C or T] is replaced by the other, the substitution is called a **transition**. If a purine is replaced by a pyrimidine or vice-versa, the substitution is called a **transversion**.)

Missense mutations

With a missense mutation, the new nucleotide alters the codon so as to produce an altered amino acid in the protein product.

Deasese: Sickle Cell anemia

The replacement of A by T at the 17th nucleotide of the gene for the beta chain of hemoglobin changes the codon GAG (for glutamic acid) to GTG (which encodes valine). Thus the 6th amino acid in the chain becomes valine instead of glutamic acid.



Figure 10.1.1 Sickle Cell Mutation

Nonsense mutations

With a nonsense mutation, the new nucleotide changes a codon that specified an amino acid to one of the **STOP** codons (**TAA**, **TAG**, or **TGA**). Therefore, **translation** of the messenger RNA **transcribed** from this mutant gene will stop prematurely. The earlier in the gene that this occurs, the more truncated the protein product and the more likely that it will be unable to function.

Cystic Fibrosis

Here is a sampling of mutations that have been found in patients with **cystic fibrosis**. Each of these mutations occurs in a huge gene that encodes a protein (of 1480 amino acids) called the **c**ystic **f**ibrosis **t**ransmembrane conductance **r**egulator (**CFTR**). The protein is responsible for transporting chloride and bicarbonate ions through the plasma membrane. The gene encompasses over 188,000 base pairs on chromosome 7 embedded in which are 27 exons encoding the protein. The numbers in the mutation column represent the number of the nucleotides affected. Defects in the protein cause the various symptoms of the disease. Unlike sickle-cell disease, then, no single mutation is responsible for all cases of cystic fibrosis. People with cystic fibrosis inherit two mutant genes, but the mutations need not be the same.

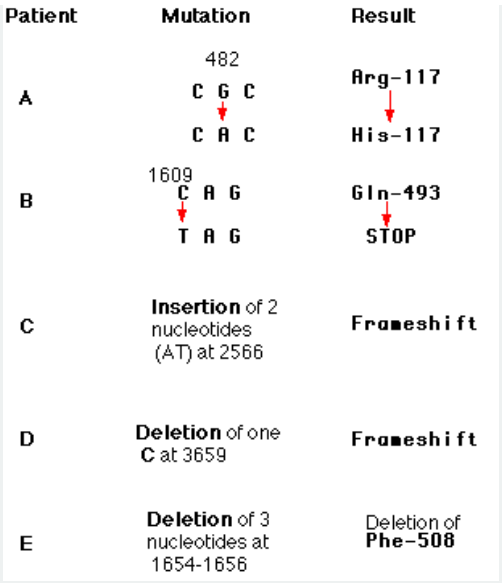


Figure 10.1.2 Cystic Fibrosis Mutation

In one patient with cystic fibrosis (**Patient B**), the substitution of a T for a C at nucleotide 1609 converted a glutamine codon (**CAG**) to a **STOP** codon (**TAG**). The protein produced by this patient had only the first 493 amino acids of the normal chain of 1480 and could not function.

Silent mutations

Most amino acids are encoded by several different codons. For example, if the third base in the **TCT** codon for **serine** is changed to any one of the other three bases, serine will still be encoded. Such mutations are said to be silent because they cause no change in their product and cannot be detected without sequencing the gene (or its mRNA).

Splice-site mutations

The removal of intron sequences, as pre-mRNA is being processed to form mRNA, must be done with great precision. Nucleotide signals at the splice sites guide the enzymatic machinery. If a mutation alters one of these signals, then the intron is not removed and remains as part of the final RNA molecule. The translation of its sequence alters the sequence of the protein product.

Insertions and Deletions (Indels)

Extra base pairs may be added (**insertions**) or removed (**deletions**) from the DNA of a gene. The number can range from one to thousands. Collectively, these mutations are called **indels**.

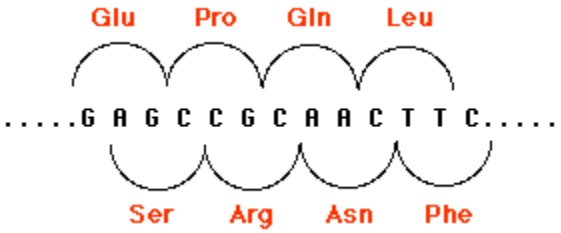


Figure 10.1.3: Frameshifting

Indels involving one or two base pairs (or multiples of two) can have devastating consequences to the gene because translation of the gene is "frameshifted". This figure shows how by **shifting the reading frame** one nucleotide to the right, the same sequence of nucleotides encodes a different sequence of amino acids. The mRNA is translated in new groups of three nucleotides and the protein specified by these new codons will be worthless. Scroll up to see two other examples (**Patients C and D**).

Frameshifts often create new **STOP** codons and thus generate nonsense mutations. Perhaps that is just as well as the protein would probably be too garbled anyway to be useful to the cell.

Indels of three nucleotides or multiples of three may be less serious because they preserve the reading frame (see the above figure).

However, a number of inherited human disorders are caused by the insertion of many copies of the same triplet of nucleotides. **Huntington's disease** and the **fragile X syndrome** are examples of such **trinucleotide repeat** diseases.

Disease: Fragile X Syndrome

Several disorders in humans are caused by the inheritance of genes that have undergone insertions of a string of 3 or 4 nucleotides repeated over and over. A locus on the human X chromosome contains such a stretch of nucleotides in which the triplet **CGG** is repeated (CGGCGGCGGCGG, etc.). The number of CGGs may be as few as 5 or as many as 50 without causing a harmful phenotype (these repeated nucleotides are in a noncoding region of the gene). Even 100 repeats usually cause no harm. However, these longer repeats have a tendency to grow longer still from one generation to the next (to as many as 4000 repeats).

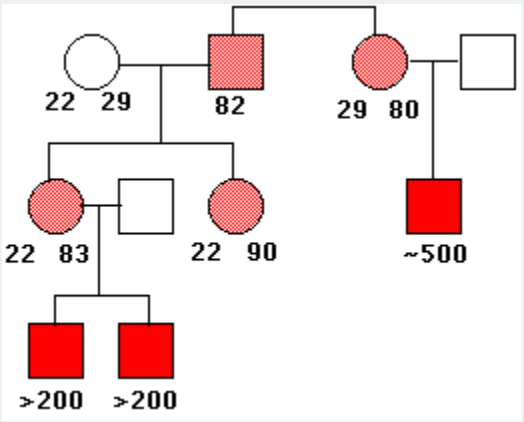


Figure 10.1.4 Fragile X Syndrome

This causes a constriction in the X chromosome, which makes it quite fragile. Males who inherit such a chromosome (only from their mothers, of course) show a number of harmful phenotypic effects including mental retardation. Females who inherit a fragile X (also from their mothers; males with the syndrome seldom become fathers) are only mildly affected.

The above image shows the pattern of inheritance of the fragile X syndrome in one family. The number of times that the trinucleotide **CGG** is repeated is given under the symbols. The gene is on the X chromosome, so women (circles) have two copies of it; men (squares) have only one. People with a gene containing 80–90 repeats are normal (light red), but this gene is unstable, and the number of repeats can increase into the hundreds in their offspring. Males who inherit such an enlarged gene suffer from the syndrome (solid red squares). (Data from C. T. Caskey, et al.).

Polyglutamine Diseases

In these disorders, the repeated trinucleotide is **CAG**, which adds a string of glutamines (Gln) to the encoded protein. These have been implicated in a number of central nervous system disorders including

- **Huntington's disease** (where the protein called **huntingtin** carries the extra glutamines). The abnormal protein increases the level of the p53 protein in brain cells causing their death by apoptosis.
- some cases of Parkinson's disease where the extra glutamines are in the protein ataxin-2.

Muscular Dystrophy

Some forms of muscular dystrophy that appear in adults are caused by tri- or tetranucleotide, e.g. (CTG)n and (CCTG)n, repeats

where n may run into the thousands. The huge RNA transcripts that result interfere with the alternative splicing of other transcripts in the nucleus.

Amyotrophic Lateral Sclerosis (ALS)

ALS is a neurodegenerative disorder leading to dementia and muscle weakness. (ALS is often called "Lou Gehrig's disease" after the baseball player who died from it.)

The most common mutation in ALS is an expansion of the number of repeats of the hexanucleotide GGGGCC in a gene on chromosome 9 from the normal two, or at least fewer than three dozen, to hundreds or even several thousand. Translation of both the sense and the antisense strands containing these repeats (and in all 3 reading frames; there is no ATG start codon) produces polymers with long strings of gly-ala, gly-pro, gly-arg (from the sense strand) as well as pro-ala, another pro-gly, and pro-arg from the antisense strand. These proteins, especially those containing arginine (arg) form aggregates that damage brain cells.

Duplications

Duplications are a doubling of a section of the genome. During meiosis, crossing over between sister chromatids that are out of alignment can produce one chromatid with a duplicated gene and the other (not shown) with the two genes with deletions. In the case shown here, unequal crossing over created a second copy of a gene needed for the synthesis of the steroid hormone **aldosterone**.

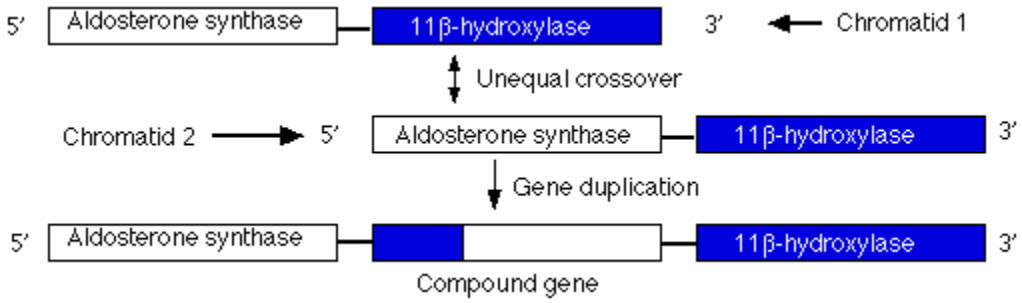


Figure 10.1.5 Gene Duplication

However, this new gene carries inappropriate promoters at its 5' end (acquired from the 11-beta hydroxylase gene) that cause it to be expressed more strongly than the normal gene. The mutant gene is dominant: all members of one family (through four generations) who inherited at least one chromosome carrying this duplication suffered from high blood pressure and were prone to early death from stroke.

Gene duplication has also been implicated in several human neurological disorders.

Gene duplication has occurred repeatedly during the evolution of eukaryotes. Genome analysis reveals many genes with similar sequences in a single organism. Presumably these paralogous genes have arisen by repeated duplication of an ancestral gene.

Such gene duplication can be beneficial.

- Over time, the duplicates can acquire different functions.
  - The proteins they encode can take on different functions; for example, if the original gene product carried out two different functions (see "pleiotropy"), each duplicated gene can now specialize at one function and do a better job at it than the parental gene.
  - But even if they do not, changes in the regulatory sequences of the genes (promoters and enhancers) may cause the same protein to be expressed at different times, at different levels, and/or in different tissues.

  Either situation can provide the basis for adaptive evolution.
- But even while two paralogous genes are still similar in sequence and function, their existence provides **redundancy** ("belt and suspenders"). This may be a major reason why knocking out genes in yeast, "knockout mice", etc. so often has such a mild effect on the phenotype. The function of the knocked out gene can be taken over by a paralog.
- After gene duplication, random loss — or inactivation — of one of these genes at a later time in
  - one group of descendants
  - different from the loss in another group

could provide a barrier (a "post-zygotic isolating mechanism") to the two groups interbreeding. Such a barrier could cause speciation: the evolution of two different species from a single ancestral species.

Translocations

Translocations are the transfer of a piece of one chromosome to a **nonhomologous chromosome**. Translocations are often reciprocal; that is, the two nonhomologues swap segments.

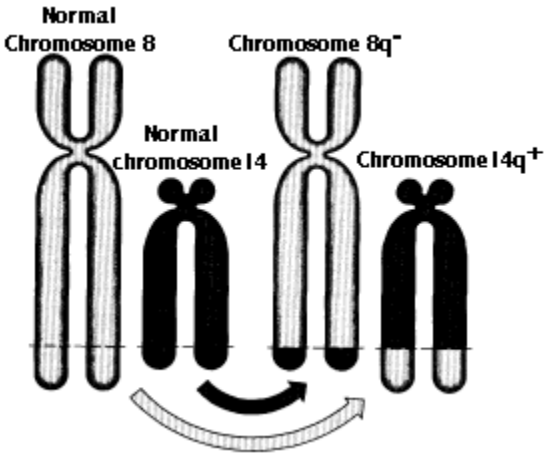


Figure 10.1.6 Translocations

Translocations can alter the phenotype is several ways:

- the break may occur **within** a gene destroying its function
- translocated genes may come under the influence of different promoters and enhancers so that their expression is altered. The t(8;14) translocation in Burkitt's lymphoma (figure) is an example.
- the breakpoint may occur **within a gene** creating a hybrid gene. This may be transcribed and translated into a protein with an N-terminal of one normal cell protein coupled to the C-terminal of another. The Philadelphia chromosome found so often in the leukemic cells of patients with **chronic myelogenous leukemia** (**CML**) is the result of a translocation which produces a compound gene (**bcr-abl**).

Frequency of Mutations

Mutations are rare events. This is surprising. Humans inherit 3×10^9 base pairs of DNA from each parent. Just considering single-base substitutions, this means that each cell has 6 billion (6×10^9) different base pairs that can be the target of a substitution. Single-base substitutions are most apt to occur when DNA is being copied; for eukaryotes that means during S phase of the cell cycle.

No process is 100% accurate. Even the most highly skilled typist will introduce errors when copying a manuscript. So it is with DNA replication. Like a conscientious typist, the cell does proofread the accuracy of its copy. But, even so, errors slip through. It has been estimated that in humans and other mammals, uncorrected errors (= mutations) occur at the rate of about 1 in every 50 million (5×10^7) nucleotides added to the chain. (Not bad — I wish that I could type so accurately.) But with 6×10^9 base pairs in a human cell, that means that each new cell contains some 120 new mutations.

Should we be worried? The evidence is not clear. Only 1.2% of our DNA encodes the exons of our proteome, and for a long time it was thought that much of the rest was "junk" DNA. Mutations in it would most likely be harmless. And even in coding regions, the existence of synonymous codons could result in the altered (mutated) gene still encoding the same amino acid in the protein. But it now appears that as much as 80% of our DNA seems to participate in regulating which genes are expressed, and how strongly, in each of the multitude of differentiated cell types in our body as each responds to the signals (nutrients, hormones, etc.) it receives. So mutations in these regions might well have harmful, if subtle, effects.

As more vertebrate genomes are sequenced, it turns out that some of these stretches of DNA that do not encode proteins none-the-less have been remarkably conserved during vertebrate evolution. Some of these regions have accumulated even fewer mutations than protein-encoding genes have. This suggests that these sequences are extremely important to the welfare of the organism. However, other regions of the genome seem able to sustain point mutations with no detectible harm.

Recent advances have enabled the coding portions of the genome of **single cells** to be sequenced. Preliminary results indicate that each **normal cell** in an adult has accumulated ~20 somatic mutations, and that its collection of mutations differs from cell to cell. **Cancer cells** accumulate many more mutations (often in the hundreds).

How can we measure the frequency at which phenotype-altering mutations occur? In humans, it is not easy.

- First we must be sure that the mutation is **newly-arisen**. (Some populations have high frequencies of a particular mutation, not because the gene is especially susceptible, but because it has been passed down through the generations from a early "founder".
- Recessive mutations (most of them are) will not be seen except on the rare occasions that both parents contribute a mutation at the same locus to their child.
- This leaves us with estimating mutation frequencies for genes that are inherited as
  - **autosomal dominants**
  - **X-linked recessives**; that is, recessives on the X chromosome which will be expressed in males because they inherit only one X chromosome.

Examples

Frequency is expressed as the frequency of mutations occurring at that locus in the gametes

- **Autosomal dominants**
  - **Retinoblastoma**

    in the ***RB*** gene: about 8 per million (8×10^{-6})
  - **Osteogenesis imperfecta**

    in one or the other of the two genes that encode **Type I collagen**: about 1 per 100,000 (10^{-5})
  - Inherited tendency to polyps (and later cancer) in the colon.

    in a tumor suppressor gene (*APC*): ~10^{-5}
- **X-linked recessives**
  - Hemophilia A

    ~3×10^{-5} (the **Factor VIII** gene)
  - Duchenne Muscular Dystrophy (DMD)

    >8×10^{-5} (the **dystrophin** gene)
    Why should the mutation frequency in the dystrophin gene be so much larger than most of the others? It's probably a matter of size. The dystrophin gene stretches over 2.4×10^{6} base pairs of DNA. This is almost 0.1% of the entire human genome! Such a huge gene offers many possibilities for damage.

Measuring Mutation Rate

The **frequency** with which a given mutation is seen in a population (e.g., the mutation that causes cystic fibrosis) provides only a rough approximation of mutation **rate** — the rate at which fresh mutations occur — because of historical factors at work such as *natural selection* (positive or negative), *drift*, and *founder effect*. In addition, most methods for counting mutations require that the mutation have a visible effect on the phenotype. Thus

- many (but not all) mutations in noncoding DNA
- mutations that produce
  - synonymous codons (encode the same amino acid)
  - or, sometimes, new codons that encode a chemically-similar amino acid
- mutations which disrupt a gene whose functions are redundant; that is, can be compensated for by other genes

will not be seen. But now these problems have been largely solved. The story is told in a report by D. R. Denver, *et al.* in the 5

August 2004 issue of **Nature**.

C. elegans

The Procedure

- Their organism = *C. elegans*
- Its advantages
  - compact genome
  - hermaphroditic — it fertilizes its own eggs and any new germline mutation will soon be either lost or appear on both homologous chromosomes.
  - rapid generation time (4 days)
- They created 198 different experimental lines of worms.
- They grew them under optimum conditions to minimize any effects of natural selection.
- Only one offspring was kept at each new generation.
- Each line was maintained for several hundred generations.
- At the end of this time, random stretches of DNA
  - derived from multiple locations on each of the six *C. elegans* chromosomes and
  - totalling an average of ~21 thousand base pairs for each line

  were sequenced from each of the 198 lines and the sequences compared with the same loci in natural populations of *C. elegans*.

Results

Examining the DNA sequences from their experimental animals (a total of over 4 million base pairs!), and comparing them with the controls, turned up a total of 30 mutations.

- 17 of these were insertions or deletions ("indels')
  - 7 in exons — all but 2 of which produced frameshifts and a premature STOP codon.
  - 10 in introns or between genes
- 13 of these were single base substitutions ("point" mutations)
  - 3 in exons : one "silent" producing a synonymous codon; two that changed the encoded amino acid.
  - 10 in introns or between genes

Calculating Mutation Rate

From these results I have pooled their data to calculate an approximate rate at which spontaneous mutations occur throughout the genome.

Mutation Rate = # of mutations observed [30] ÷ (# of experimental lines [198]) x (average # of generations [339]) x (average # of base pairs sequenced [~21,000])

yielding a rate of 2.1×10^{-8} mutations per base pair per generation.

The total *C. elegans* genome contains some 10^8 base pairs so this tells us that **two** new germline mutations occur somewhere in each of *C. elegans*'s two haploid genomes in each generation.

A similar analysis for Drosophila (whose genome is about the same size as that of *C. elegans*) showed a similar mutation rate: ~10^{-8} mutations per base pair per generation. As for the green plant *Arabidopsis thaliana*, its spontaneous mutation rate is slightly lower: ~7×10^{-9} mutations per base pair per generation.

In the 30 April 2010 issue of **Science**, Roach, J. C., *et al*., reported that the rate for **humans** is in the same range: ~1.1×10^{-8} mutations per base pair in the haploid genome. With a diploid genome of 6×10^9 base pairs, that works out to some 70 new mutations in each child. They derived these numbers from comparing the complete genome sequence of two children and their parents.

> In the 20 July 2012 issue of **Cell**, Wang, J., *et al*. reported the results of sequencing 8 individual sperm cells from a 40-year-old man. They found a mutation rate ranging from 2.0×10^{-8} to 3.8×10^{-8}.
>
> Should we be worried about such spontaneous mutation rates? Probably not too much. With our high proportion of noncoding DNA, many mutations will occur in regions that will have no effect on our phenotype. Evidence: out of a total of 251 mutations found in the 8 sperm cells, only 3 were missense mutations altering a gene product. However, even in noncoding DNA, point mutations may affect the expression of genes, so perhaps as many as 10% of the point mutations a child inherits may have harmful, if subtle, effects.

Males Contribute More Mutations Than Females

If most mutations occur during S phase of cell division, then males should be more at risk. This is because only two dozen (24) or so mitotic divisions occur from the fertilized egg that starts a little girl's embryonic development and the setting aside of her future eggs (which is done long before she is even born). Furthermore, the sperm of a 30-year old man, in contrast, are the descendants of at least 400 mitotic divisions since the fertilized egg that formed him.

So, fathers are more likely than mothers to transmit newly-formed mutations to their children. The sperm of a 25-year-old man might carry some 45 new mutations. This number rises at a rate of about 1 per year, so the sperm of a 40-year-old man may transmit some 60 new mutations to his children (about 20 of these in coding regions). No matter what the age of the mother, she transmits only about 15 new mutations to her offspring. (But **chromosomal aberrations**, like aneuploidy, are more apt to arise in eggs than in sperm, and the incidence of these increases with maternal age.) These data explain why the children of aged fathers suffer more genetic disorders than those of young fathers.

Somatic vs. Germline Mutations

The significance of mutations is profoundly influenced by the distinction between germline and soma. Mutations that occur in a **somatic cell**, in the bone marrow or liver for example, may

- damage the cell
- make the cell cancerous
- kill the cell

Whatever the effect, the ultimate fate of that **somatic mutation** is to disappear when the cell in which it occurred, or its owner, dies.

Germline mutations, in contrast, will be found in every cell descended from the zygote to which that mutant gamete contributed. If an adult is successfully produced, every one of its cells will contain the mutation. Included among these will be the next generation of gametes, so if the owner is able to become a parent, that mutation will pass down to yet another generation.

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10.2: Testing for Mutagenic Chemicals in Bacteria and Mice

Ames test

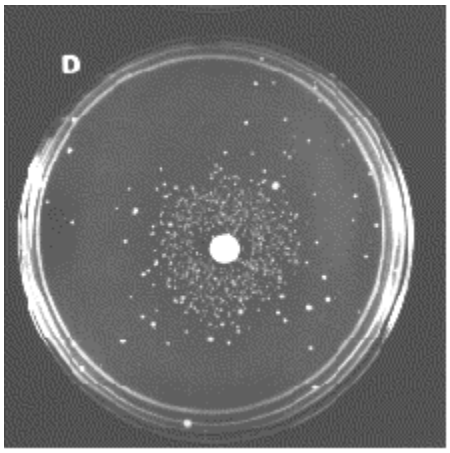


Figure 10.2.2 Blue Mouse

Big Blue mice are transgenic for a segment of DNA that contains the DNA of **bacteriophage lambda**, a virus that infects **E. coli**, and which serves here as the vector for 3 genetic elements from the **lac operon** of *E. coli:*

- the *lacI* gene
- the **operator** of the operon
- the beta-galactosidase (*lacZ*) gene

The Assay

The transgenic mice are given repeated doses of the suspected carcinogen for a week or two. If the chemical is mutagenic, it will cause random mutations throughout the genome of each mouse cell. If a mutation occurs in either the **lacI** gene (which encodes the **lac repressor**) or the operator,

the gene (*lacZ*) for beta-galactosidase will **no longer be repressed**. To detect this,

- The DNA is extracted from the tissues of the treated mouse.
- The vector is isolated and used to make functional bacteriophages.
- E. coli cells are mixed with the bacteriophage and spread on a solid culture medium.
- The bacteriophages infect and destroy ("lyze") the *E. coli* cells.
- This causes clear circular zones, called **plaques**, to appear in a "lawn" of bacteria.
- Before they die, cells that have been infected by bacteriophages carrying a mutated ***lacI*** or **operator** will produce beta-galactosidase.
- This reacts with a substrate in the culture medium turning it blue.
- Bacteriophages with unmutated genes produce colorless plaques because no beta-galactosidase is synthesized.
- Count both colorless and blue plaques.
- The number of blue plaques divided by the total number of plaques gives the **mutation frequency**.

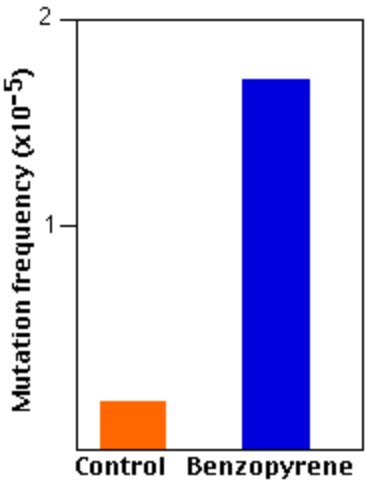


Figure 10.2.4 Blue Plaque courtesy of Stratagene

This photograph shows one mutant (blue) plaque on a lawn of *E. coli* containing many non-mutant (clear) plaques.

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10.3: Radiation and its effect on DNA

For biologists, the most significant forms of radiation are light, heat, and ionizing radiation. **Ionizing radiation** can penetrate cells and create ions in the cell contents. These, in turn, can cause permanent alterations in DNA (i.e., mutations). Ionizing radiation includes X rays, gamma rays, neutrons, electrons ("beta" particles), and alpha particles (helium nuclei).

Units of measurement

- rad: The rad represents a certain dose of energy absorbed by 1 gram of tissue. It is a unit of concentration. So if we could uniformly expose the entire body to radiation, the number of rads received would be the same whether we were speaking of a single cell, an organ (e.g., an ovary) or the entire body (just as the concentration of salt in sea water is the same whether we consider a cupful or an entire ocean).
- rem: Some forms of radiation are more efficient than others transferring their energy to the cell. To have a level playing field, it is convenient to multiply the dose in rads by a **quality factor** (**Q**) for each type of radiation. The resulting unit is the **rem** ("**r**oentgen-**e**quivalent **m**an"). Thus, **rem = rad x Q**. X rays and gamma rays have a Q about 1, so the absorbed dose in rads is the same number in rems. Neutrons have a Q of about 5 and alpha particles have a Q of about 20. An absorbed dose of, say, 1 rad of these is equivalent to 5 rem and 20 rem respectively.
- The sievert (Sv) and gray (Gy): Despite the years of high-quality research reported in rems and millirems (mrem, 10^{-3} rem), the International Commission on Radiation Units and Measurements wants us to give up the rad in favor of the gray (Gy), a unit 100 times larger. Similarly, the rem is to be replaced by the **sievert** (**Sv**), again so that 100 rem = 1 Sv. So I will try to express all radiation doses in a single unit, the **millisievert** (**mSv**).

Table 10.2.1: An assortment of typical radiation doses (in mSv)

Used to destroy the bone marrow in preparation for a marrow transplant (given over several days)	10,000
Approximate lethal dose ("LD50") if no treatment and given to the entire body in a short period	4,500
Causes radiation sickness (when absorbed in a short period)	>1,000
When delivered in a single dose, increases the risk of developing cancer by 1%	100
Increase in lifetime dose to most heavily exposed people living near Chernobyl	430
Annual dose (excluding natural background) permitted for U.S. radiation workers	50
Average annual dose (excluding natural background) for medical X-ray technicians	3.2
Maximum permissible annual dose (excluding natural background and medical exposure) to general public	1.7
Average annual dose of natural background radiation, worldwide	2.4
Natural background, Boston, MA, USA (per year)(excluding radon)	1.02
Natural background, Denver, CO, USA (per year)(excluding radon)	1.8
Additional annual dose if you live in a brick rather than a wood house	0.07
Annual dose in some houses in Ramsar, Iran	>130
Average dose to person living within 10 miles of Three-Mile Island (TMI) caused by the accident of 28 March 1979	0.01
Most heavily exposed person (a fisherman) near TMI	<1.0
Approximate dose received by a person spending 1 year at the fence surrounding a nuclear power station	0.001–0.006
Average dose to each person in the U. S. population from nuclear power plants (per year)	0.00002
Received by the brain during a set of dental x rays	0.005

Received by the colon during a barium enema	15
Received by the lungs during a chest x ray	0.01—0.15
Screening mammogram	0.5
Total dose received by the people living near the Fukushima Daiichi Nuclear Power Station in Japan during the first year after the reactors were damaged by a devastating tsunami.	12–25
Dose from a typical set of full-body computed tomography (CT) scans	20
Cardiac stress test using radioactive thallium	36
Typical dose received by the abdomen during a CT scan to diagnose appendicitis	10
Typical PET scan	14.0
Airline passenger crossing the U.S.	0.04
Flight crew flying regularly between New York and Tokyo (per year)	9
Hourly dose to skin holding piece of the original "Fiesta Ware" (a brand of pottery)	2–3
Annual dose to each person in the U. S. population from fallout (former weapons testing plus Chernobyl)	0.0006

Estimated average annual radiation exposure from various sources (in millisieverts) of an inhabitant of the United States (total = 5.86 mSv). Individual exposures, especially to radon and medical sources, vary widely from these average values. The use of medical imaging in the United States (some 67 million CT scans were performed here in 2006) has increased greatly in recent years. As for radon, only the lungs are exposed as the alpha particles emitted by radon cannot penetrate other tissues. (Data from the National Council on Radiation Protection and Measurements, Bethesda, MD.)

Background Radiation

About 27% of our annual exposure to radiation is from background radiation (Figure 10.3.1) and originate from three primary sources:

1. Cosmic radiation (0.27 mSv). The value increases with altitude, so the dose for people in Denver, Colorado is about 0.50 mSv.
2. Rocks and soil (0.28 mSv). This value varies with the geology of a region: people in Louisiana get as little as 0.15 mSv/yr; people on the Colorado plateau (incl. Denver!) get 1.4 mSv/yr.
3. From within the body (0.4 mSv). Most of this comes from potassium-40. About 0.02% of the potassium in nature is in the form of the radioactive isotope ^{40}K. Living tissue cannot discriminate between radioactive and nonradioactive versions, so the same 0.02% of the total potassium in the body (about 1.7 g in a 70-kg person) is radioactive.

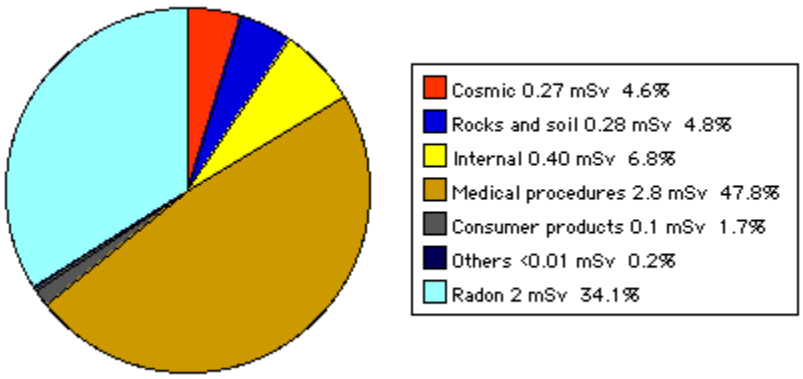


Figure 10.3.1 Radiation Pie Chart

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10.4: Transposons - "jumping genes"

Transposons are segments of DNA that can move around to different positions in the genome of a single cell. In the process, they may cause mutations and increase (or decrease) the amount of DNA in the genome of the cell, and if the cell is the precursor of a gamete, in the genomes of any descendants. These mobile segments of DNA are sometimes called **"jumping genes"** and there are two distinct types. **Class II transposons** consist of DNA that moves directly from place to place. **Class I transposons** are **retrotransposons** that first transcribe the DNA into RNA and then use reverse transcriptase to make a DNA copy of the RNA to insert in a new location.

Class II Transposons

Class II transposons move by a "**cut and paste**" process: the transposon is cut out of its location (like **command/control-X** on your computer) and inserted into a new location (**command/control-V**). This process requires an enzyme — a **transposase** — that is encoded within some of these transposons.

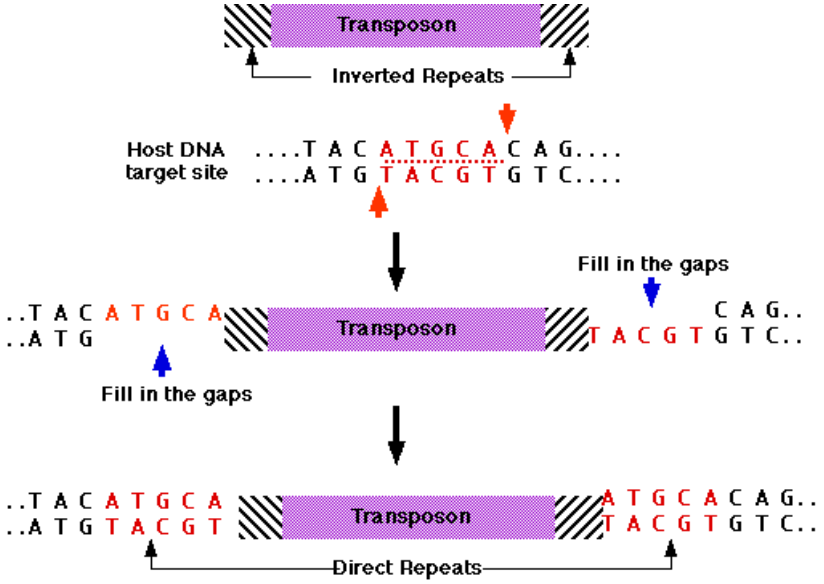


Figure 10.4.1 Transposons

Transposase binds to both ends of the transposon, which consist of **inverted repeats**; that is, identical sequences reading in opposite directions. They also bind to a sequence of DNA that makes up the target site. Some transposases require a specific sequence as their target site; others can insert the transposon anywhere in the genome.

The DNA at the target site is cut in an offset manner (like the "sticky ends" produced by some restriction enzymes). After the transposon is ligated to the host DNA, the gaps are filled in by Watson-Crick base pairing. This creates identical **direct repeats** at each end of the transposon. Often transposons lose their gene for transposase. However, as long as somewhere in the cell there is a transposon that can synthesize the enzyme, their inverted repeats are recognized and they, too, can be moved to a new location.

Miniature Inverted-repeat Transposable Elements (MITEs)

The recent completion of the genome sequence of rice and *C. elegans* has revealed that their genomes contain thousands of copies of a recurring motif consisting of almost identical sequences of about 400 base pairs flanked by characteristic **inverted repeats** of about 15 base pairs such as

5' GGCCAGTCACAATGG..~400 nt..CCATTGTGACTGGCC 3'

3' CCGGTCAGTGTTACC..~400 nt..GGTAACACTGACCGG 5'

MITEs are too small to encode any protein. Just how they are copied and moved to new locations is still uncertain. Probably larger transposons that do encode the necessary enzyme and recognize the same inverted repeats are responsible. There are over 100,000 MITEs in the rice genome (representing some 6% of the total genome). Some of the **mutations** found in certain strains of rice are caused by the insertion of a MITE in the gene. MITEs have also been found in the genomes of humans, Xenopus, and apples.

Transposons in Maize

The first transposons were discovered in the 1940s by Barbara McClintock who worked with maize (*Zea mays*, called "corn" in the U.S.). She found that they were responsible for a variety of types of **gene mutations**, usually insertions and deletions (indels) and translocations. Some of the mutations (*c*, *bz*) used as examples of how gene loci are mapped on the chromosome were caused by transposons. In developing somatic tissues like corn kernels, a mutation (e.g., *c*) that alters color will be passed on to all the descendant cells. This produces the variegated pattern which is so prized in "Indian corn". (Photo courtesy of Whalls Farms.) It took about 40 years for other scientists to fully appreciate the significance of Barbara McClintock's discoveries. She was finally awarded a Nobel Prize in 1983.



Figure 10.4.2: Transposons in Maize

Transposons in Drosophila

P elements are **Class II transposons** found in *Drosophila*. They do little harm because expression of their transposase gene is usually repressed. However, when male flies with P elements mate with female flies lacking them, the transposase becomes active in the germline producing so many mutations that their offspring are sterile. In nature this is no longer a problem. P elements seem to have first appeared in *Drosophila melanogaster* about 50 years ago. Since then, they have spread through every population of the species. Today flies lacking P elements can only be found in old strains maintained in the laboratory. P elements have provided valuable tools for Drosophila geneticists. Transgenic flies containing any desired gene can be produced by injecting the early embryo with an engineered P element containing that gene. Other transposons are being studied for their ability to create transgenic insects of agricultural and public health importance.

Transposons in bacteria

Some transposons in bacteria carry — in addition to the gene for transposase — genes for one or more (usually more) proteins imparting resistance to antibiotics. When such a transposon is incorporated in a plasmid, it can leave the host cell and move to another. This is the way that the alarming phenomenon of multidrug antibiotic resistance spreads so rapidly. Transposition in these cases occurs by a "**copy and paste**" mechanism. This requires an additional enzyme — a **resolvase** — that is also encoded in the transposon itself. The original transposon remains at the original site while its copy is inserted at a new site.

Retrotransposons

Retrotransposons also move by a "**copy and paste**" mechanism but in contrast to the transposons described above, the copy is made of RNA, not DNA. The RNA copies are then transcribed back into DNA — using a reverse transcriptase — and these are inserted into new locations in the genome. Many retrotransposons have **long terminal repeats** (**LTRs**) at their ends that may contain over 1000 base pairs in each. Like DNA transposons, retrotransposons generate direct repeats at their new sites of insertion. In fact, it is the presence of these direct repeats that often is the clue that the intervening stretch of DNA arrived there by retrotransposition. Some 50% of the entire human genome consists of retrotransposons.

LINEs (Long interspersed elements)

The human genome contains over one million LINEs (representing 19% of the genome). The most abundant of these belong to a family called LINE-1 (L1). These L1 elements are DNA sequences that range in length from a few hundred to as many as 9,000 base pairs. Only about 50 L1 elements are functional "genes"; that is, can be transcribed and translated. The functional L1 elements are about 6,500 bp in length and encode three proteins, including an endonuclease that cuts DNA and a reverse transcriptase that makes a DNA copy of an RNA transcript.

L1 activity

L1 activity proceeds as follows:

1. RNA polymerase II transcribes the L1 DNA into RNA.
2. The RNA is translated by ribosomes in the cytoplasm into the proteins.
3. The proteins and RNA join together and reenter the nucleus.
4. The endonuclease cuts a strand of "target" DNA, often in the intron of a gene.
5. The reverse transcriptase copies the L1 RNA into L1 DNA which is inserted into the target DNA forming a new L1 element there.

Through this **copy-paste** mechanism, the number of LINEs can increase in the genome. The diversity of LINEs between individual human genomes make them useful markers for DNA "fingerprinting". Variation occurs in the length of L1 elements: Transcription of an active L1 element sometimes continues downstream into additional DNA producing a longer transposed element. **Reverse** transcription of L1 RNA often concludes prematurely and produces a shortened transposed element.

While L1 elements are not functional, they may play a role in regulating the efficiency of transcription of the gene in which they reside. Occasionally, L1 activity makes and inserts a copy of a cellular mRNA (thus a natural cDNA). Lacking introns as well as the necessary control elements like promoters, these genes are not expressed. They represent one category of **pseudogene**.

SINEs (Short interspersed elements)

SINEs are short DNA sequences (100–400 base pairs) that represent **reverse-transcribed RNA** molecules originally transcribed by RNA polymerase III; that is, molecules of tRNA, 5S rRNA, and some other small nuclear RNAs. The most abundant SINEs are the **Alu elements**. There are over one million copies in the human genome (representing 9% of our total DNA). Alu elements consist of a sequence averaging 260 base pairs that contains a site that is recognized by the restriction enzyme **AluI**. They appear to be reverse transcripts of 7S RNA, part of the signal recognition particle. Most SINEs do not encode any functional molecules and depend on the machinery of active L1 elements to be transposed; that is, copied and pasted in new locations.

HIV-1

HIV-1 — the cause of AIDS — and other human retroviruses (e.g., HTLV-1, the human T-cell leukemia/lymphoma virus) behave like retrotransposons. The RNA genome of HIV-1 contains a gene for **reverse transcriptase** and one for **integrase**. The integrase serves the same function as the transposases of DNA transposons. The DNA copies can be inserted anywhere in the genome. Molecules of both enzymes are incorporated in the virus particle.

Transposons and Mutations

Transposons are **mutagens** and can cause mutations in several ways. If a transposon inserts itself into a functional gene, it will probably damage it. Insertion into exons, introns, and even into DNA flanking the genes (which may contain promoters and enhancers) can destroy or alter the gene's activity. Faulty repair of the gap left at the old site (in cut and paste transposition) can lead to mutation there. The presence of a string of identical repeated sequences presents a problem for precise pairing during meiosis. How is the third, say, of a string of five Alu sequences on the "invading strand" of one chromatid going to ensure that it pairs with the third sequence in the other strand? If it accidentally pairs with one of the other Alu sequences, the result will be an unequal crossover — one of the commonest causes of **duplications**.

Note

The insertion of a retrotransposon in the DNA flanking a gene for pigment synthesis is thought to have produced white grapes from a black-skinned ancestor. Later, the loss of that retrotransposon produced the red-skinned grape varieties cultivated today.

SINEs (mostly Alu sequences) and LINEs cause only a small percentage of human mutations. (There may even be a mechanism by which they avoid inserting themselves into functional genes.) However, they have been found to be the cause of the mutations responsible for **some** cases of human genetic diseases, including:

- Hemophilia A (**Factor VIII** gene) and Hemophilia B [**Factor IX** gene]
- X-linked severe combined immunodeficiency (SCID) [gene for part of the IL-2 receptor]
- porphyria
- predisposition to colon polyps and cancer [**APC** gene]
- Duchenne muscular dystrophy [**dystrophin** gene]

What good are transposons?

Transposons have been called "junk" DNA and "selfish" DNA. They are "selfish" because their only function seems to make more copies of themselves and "junk" because there is no obvious benefit to their host. Because of the sequence similarities of all the LINEs and SINEs, they also make up a large portion of the **"repetitive DNA"** of the cell. Retrotransposons cannot be so selfish that they reduce the survival of their host. And it now appears that many, at least, confer some benefit. The ENCODE project found that some 75% of our repetitive DNA occurs within, or overlaps with, sequences, like enhancers, that regulate gene expression.

Some other possibilities:

- Retrotransposons often carry some additional sequences at their 3' end as they insert into a new location. Perhaps these occasionally create new combinations of exons, promoters, and enhancers that benefit the host.

  Example:

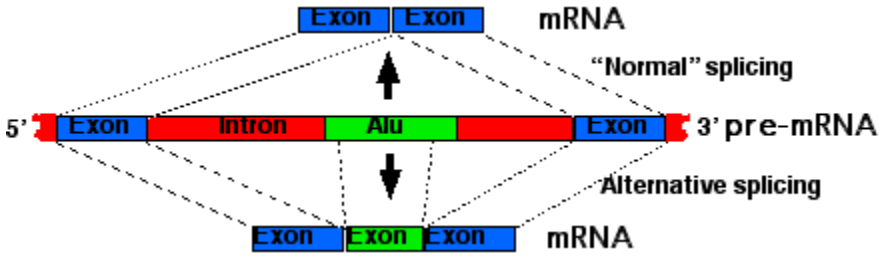


Figure 10.4.3 Retrotransposons

  - Thousands of our Alu elements occur in the introns of genes.
  - Some of these contain sequences that when transcribed into the primary transcript are recognized by the spliceosome.
  - These can then be spliced into the mature mRNA creating a new exon, which will be transcribed into a new protein product.

    - Alternative splicing can provide not only the new mRNA (and thus protein) but also the old.
    - In this way, nature can try out new proteins without the risk of abandoning the tried-and-true old one.

- L1 elements inserted into the introns of functional genes reduce the transcription of those genes without harming the gene product — the longer the L1 element, the lower the level of gene expression. Some 79% of our genes contain L1 elements, and perhaps they are a mechanism for establishing the baseline level of gene activity.
- Telomerase, the enzyme essential for maintaining chromosome length, is closely related to the reverse transcriptase of LINEs and may have evolved from it.
- *RAG-1* and *RAG-2*. The proteins encoded by these genes are needed to assemble the repertoire of antibodies and T-cells receptors (TCRs) used by the adaptive immune system. The mechanism resembles that of the cut and paste method of Class II transposons , and the RAG genes may have evolved from them. If so, the event occurred some 450 million years ago when the jawed vertebrates evolved from jawless ancestors. Only jawed vertebrates have the *RAG-1* and *RAG-2* genes.
- In *Drosophila*, the insertion of transposons into genes has been linked to the development of resistance to DDT and organophosphate insecticides.

Transposons and the C-value Paradox

The genome of *Arabidopsis thaliana* contains ~1.2 x 10^8 base pairs (bp) of DNA. About 14% of this consists of transposons; the rest functional genes (25,498 of them). The maize (corn) genome contains 20 times more DNA (2.4 x 10^9 bp) but surely has no need for 20 times as many genes. In fact, 60% of the corn genome is made up of transposons (the figure for humans is 42%). So it seems likely that the lack of an association between size of genome and number of functional genes — the C-value paradox — is caused by the amount of transposon DNA accumulated in the genome.

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UNIT 11: GENOMICS

11.1: Recombinant DNA and Gene Cloning

11.2: Polymerase Chain Reaction

11.3: Gene Therapy - Methods and Prospects

11.4: Recent Advances in Gene Therapy

11.5: Transgenic Animals

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11.11: Antisense Oligodeoxynucleotides and their Therapeutic Potential

11.12: Forward and Reverse genetics

11.13: Metagenomics

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11.1: Recombinant DNA and Gene Cloning

Recombinant DNA is DNA that has been created artificially. DNA from two or more sources is incorporated into a single recombinant molecule.

Making Recombinant DNA (rDNA) - An Overview

- Treat DNA from both sources with the same restriction endonuclease (BamHI in this case).
- BamHI cuts the same site on both molecules

  5' GGATCC 3'
  3' CCTAGG 5'

- The ends of the cut have an overhanging piece of single-stranded DNA.
- These are called "sticky ends" because they are able to base pair with any DNA molecule containing the complementary sticky end.
- In this case, both DNA preparations have complementary sticky ends and thus can pair with each other when mixed.
- A **DNA ligase** covalently links the two into a molecule of **recombinant DNA**.

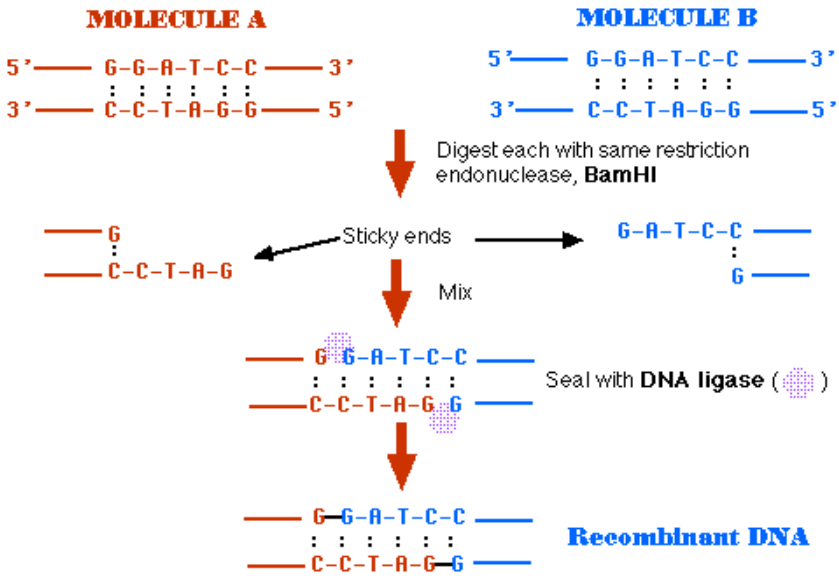


Figure 11.1.1 Making a rDNA

To be useful, the recombinant molecule must be replicated many times to provide material for analysis, sequencing, etc. Producing many identical copies of the same recombinant molecule is called **cloning**. Cloning can be done in vitro, by a process called the polymerase chain reaction (PCR). Here, however, we shall examine how cloning is done *in vivo*.

Cloning in vivo can be done in

- unicellular microbes like ***E. coli***
- unicellular eukaryotes like **yeast** and
- in mammalian cells grown in tissue culture.

In every case, the recombinant DNA must be taken up by the cell in a form in which it can be replicated and expressed. This is achieved by incorporating the DNA in a **vector**. A number of viruses (both bacterial and of mammalian cells) can serve as vectors. But here let us examine an example of cloning using ***E. coli*** as the host and a **plasmid** as the vector.

Plasmids

Plasmids are small (a few thousand base pairs), usually carry only one or a few genes, are circular and have a single **origin of replication.** Plasmids are replicated by the same machinery that replicates the bacterial chromosome. Some plasmids are copied at about the same rate as the chromosome, so a single cell is apt to have only a single copy of the plasmid. Other plasmids are copied at a high rate and a single cell may have 50 or more of them.

Genes on plasmids with high numbers of copies are usually expressed at high levels. In nature, these genes often encode proteins (e.g., enzymes) that protect the bacterium from one or more **antibiotics**. Plasmids enter the bacterial cell with relative ease. This occurs in nature and may account for the rapid spread of antibiotic resistance in hospitals and elsewhere. Plasmids can be deliberately introduced into bacteria in the laboratory transforming the cell with the incoming genes.

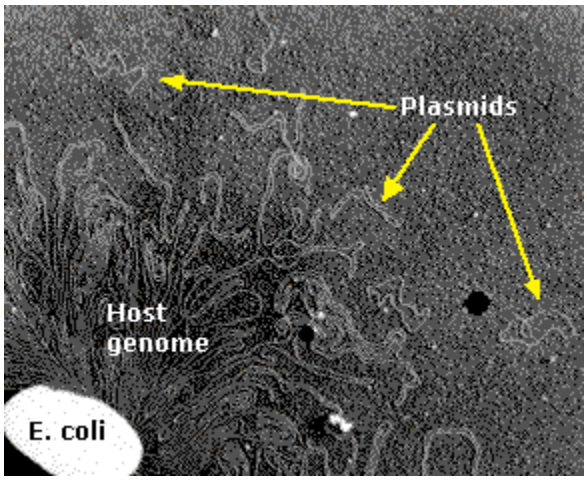


Figure 11.1.2 Electron micrograph of an E. coli cell ruptured to release its DNA Courtesy of Huntington Potter and David Dressler, Harvard Medical School

In the above figure, the tangle is a portion of a single DNA molecule containing over 4.6 million base pairs encoding approximately 4,300 genes. The small circlets are plasmids.

Examples of Plasmids

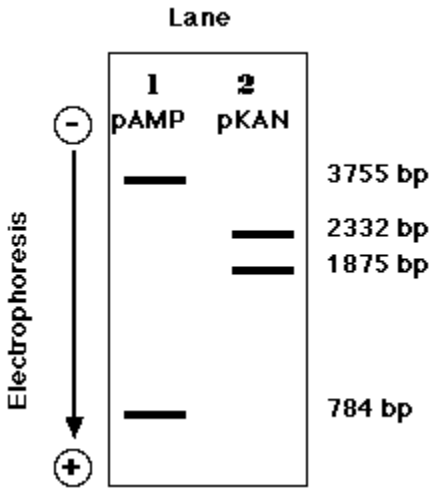


Figure 11.1.3 pAMP/ pKAN

pAMP

- 4539 base pairs
- a single replication origin

- a gene (**amp^r**)conferring resistance to the antibiotic **ampicillin** (a relative of penicillin)
- a **single** occurrence of the sequence

  5' GGATCC 3'
  3' CCTAGG 5'that, as we saw above, is cut by the restriction enzyme **BamHI**

- a **single** occurrence of the sequence

  5' AAGCTT 3'
  3' TTCGAA 5'that is cut by the restriction enzyme **HindIII**

Treatment of pAMP with a **mixture** of BamHI and HindIII produces:

- a fragment of **3755** base pairs carrying both the **amp^r** gene and the replication origin
- a fragment of **784** base pairs
- both fragments have sticky ends

pKAN

- 4207 base pairs
- a single replication origin
- a gene (**kan^r**) conferring resistance to the antibiotic **kanamycin**.
- a single site cut by **BamHI**
- a single site cut by **HindIII**

Treatment of pKAN with a **mixture** of BamHI and HindIII produces:

- a fragment of **2332** base pairs
- a fragment of **1875** base pairs with the **kan^r** gene (but no origin of replication)
- both fragments have sticky ends

These fragments can be visualized by subjecting the digestion mixtures to electrophoresis in an agarose gel. Because of its negatively-charged phosphate groups, DNA migrates toward the positive electrode (anode) when a direct current is applied. The smaller the fragment, the farther it migrates in the gel.

Ligation Possibilities

If you remove the two restriction enzymes and provide the conditions for DNA ligase to do its work, the pieces of these plasmids can rejoin (thanks to the complementarity of their sticky ends).

Mixing the pKAN and pAMP fragments provides several (at least 10) possibilities of rejoined molecules. Some of these will not produce functional plasmids (molecules with two or with no replication origin cannot function).

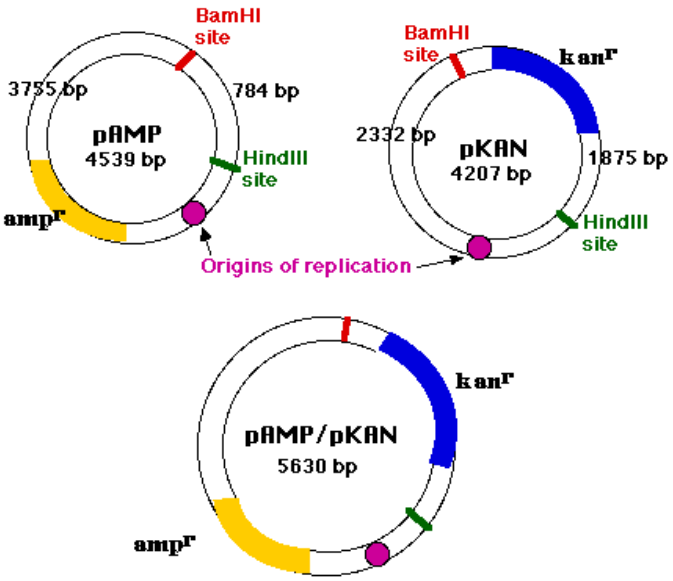


Fig.11.1.4 Recombinant Plasmid

One interesting possibility is the joining of

- the 3755-bp pAMP fragment (with **amp^r** and a replication origin) with the
- 1875-bp pKAN fragment (with **kan^r**)

Sealed with **DNA ligase**, these molecules are functioning plasmids that are capable of conferring resistance to **both** ampicillin and kanamycin. They are molecules of **recombinant DNA**.

Because the replication origin, which enables the molecule to function as a plasmid, was contributed by pAMP, pAMP is called the **vector**.

Transforming *E. coli*

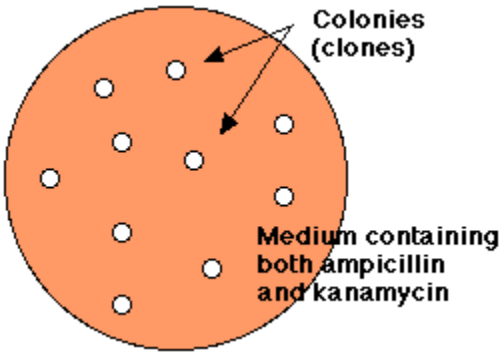


Figure 11.1.5 Colonies of E. coli

Treatment of *E. coli* with the mixture of religated molecules will produce some colonies that are able to grow in the presence of both ampicillin and kanamycin.

- A suspension of *E. coli* is treated with the mixture of religated DNA molecules.
- The suspension is spread on the surface of agar containing both ampicillin and kanamycin.
- The next day, a few cells — resistant to both antibiotics — will have grown into visible colonies containing billions of transformed cells.
- Each colony represents a **clone** of transformed cells.

However, *E. coli* can be simultaneously transformed by more than one plasmid, so we must demonstrate that the transformed cells have acquired the recombinant plasmid.

Electrophoresis of the DNA from doubly-resistant colonies (clones) tells the story.

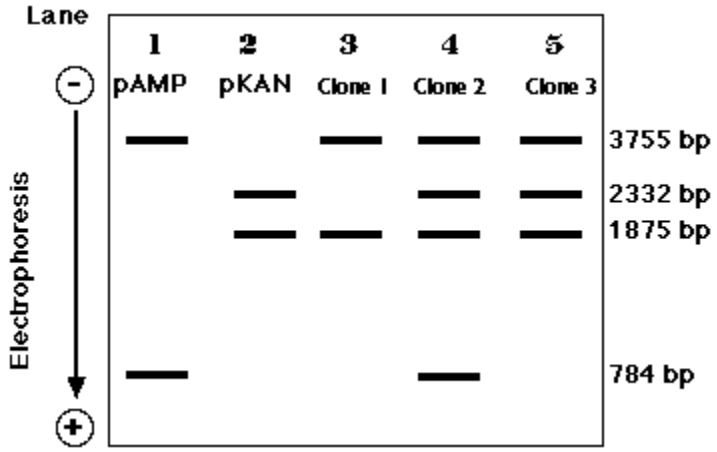


Figure 11.1.6 Cloning

- Plasmid DNA from cells that acquired their resistance from a **recombinant plasmid** only show only the **3755**-bp and **1875**-bp bands (**Clone 1**, lane 3).
- **Clone 2** (Lane 4) was simultaneous transformed by religated pAMP and pKAN. (We cannot tell if it took up the recombinant molecule as well.)
- **Clone 3** (Lane 5) was transformed by the recombinant molecule as well as by an intact pKAN.

Cloning other Genes

The recombinant vector described above could itself be a useful tool for cloning other genes. Let us assume that within its **kanamycin resistance gene** (**kan^r**) there is a single occurrence of the sequence

5' GAATTC 3'
3' CTTAAG 5'

This is cut by the restriction enzyme **EcoRI**, producing sticky ends.

If we treat any other sample of DNA, e.g., from human cells, with EcoRI, fragments with the same sticky ends will be formed. Mixed with EcoRI-treated plasmid and DNA ligase, a small number of the human molecules will become incorporated into the plasmid which can then be used to transform *E. coli*.

But how to detect those clones of *E. coli* that have been transformed by a plasmid carrying a piece of human DNA?

The key is that the EcoRI site is **within** the **kan^r** gene, so when a piece of human DNA is inserted there, the gene's function is destroyed.

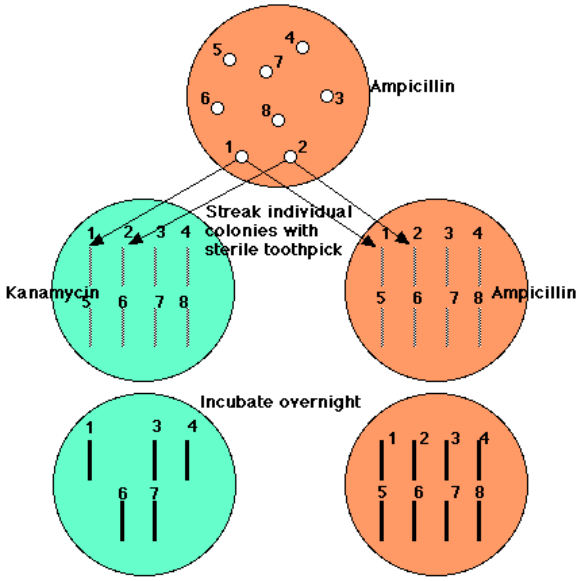


Figure 11.1.7 Screening Clones

All *E. coli* cells transformed by the vector, whether it carries human DNA or not, can grow in the presence of ampicillin. But *E. coli* cells transformed by a plasmid carrying human DNA will be unable to grow in the presence of kanamycin. So,

- Spread a suspension of treated *E. coli* on agar containing ampicillin only
- grow overnight
- with a sterile toothpick transfer a small amount of each colony to an identified spot on agar containing kanamycin
- (do the same with another ampicillin plate)
- Incubate overnight

All those clones that continue to grow on ampicillin but fail to grow on kanamycin (here, **clones 2**, **5**, and **8**) have been transformed with a piece of human DNA.

Some recombinant DNA products being used in human therapy

Using procedures like this, many human genes have been cloned in *E. coli* or in yeast. This has made it possible — for the first time — to produce unlimited amounts of human proteins in vitro. Cultured cells (*E. coli*, yeast, mammalian cells) transformed with a human gene are being used to manufacture more than 100 products for human therapy. Some examples:

- **insulin** for diabetics
- **factor VIII** for males suffering from hemophilia A
- **factor IX** for hemophilia B
- **human growth hormone** (**HGH**)
- **erythropoietin** (**EPO**) for treating anemia
- several types of **interferons**
- several **interleukins**
- **granulocyte-macrophage colony-stimulating factor** (**GM-CSF**) for stimulating the bone marrow after a bone marrow transplant
- **granulocyte colony-stimulating factor** (**G-CSF**) for stimulating neutrophil production (e.g., after chemotherapy) and for mobilizing hematopoietic stem cells from the bone marrow into the blood.
- **tissue plasminogen activator** (**TPA**) for dissolving blood clots
- **adenosine deaminase** (**ADA**) for treating some forms of **severe combined immunodeficiency** (**SCID**)
- **parathyroid hormone**
- many **monoclonal antibodies**
- **hepatitis B surface antigen** (**HBsAg**) to vaccinate against the hepatitis B virus
- **C1 inhibitor** (**C1INH**) used to treat hereditary angioedema

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11.2: Polymerase Chain Reaction

The polymerase chain reaction is a technique for quickly "cloning" a particular piece of DNA in the test tube (rather than in living cells like *E. coli*). Thanks to this procedure, one can make virtually unlimited copies of a single DNA molecule even though it is initially present in a mixture containing many different DNA molecules.

Procedure

To perform PCR, you must know at least a portion of the sequence of the DNA molecule that you wish to replicate. You must then synthesize **primers**: short oligonucleotides (containing about two dozen nucleotides) that are precisely complementary to the sequence at the 3' end of each strand of the DNA you wish to amplify. The DNA sample is heated to separate its strands and mixed with the primers. If the primers find their complementary sequences in the DNA, they bind to them; synthesis begins (as always 5' -> 3') using the original strand as the template.

The reaction mixture must contain all four deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP), and a DNA polymerase. It helps to use a DNA polymerase that is not denatured by the high temperature needed to separate the DNA strands. Polymerization continues until each newly-synthesized strand has proceeded far enough to contain the site recognized by the **other primer**. Now you have two DNA molecules identical to the original molecule. You take these two molecules, heat them to separate their strands, and repeat the process. Each cycle doubles the number of DNA molecules.

Using automated equipment, each cycle of replication can be completed in less than 5 minutes. After 30 cycles, what began as a single molecule of DNA has been amplified into more than a billion copies ($2^{30} = 1.02 \times 10^9$).

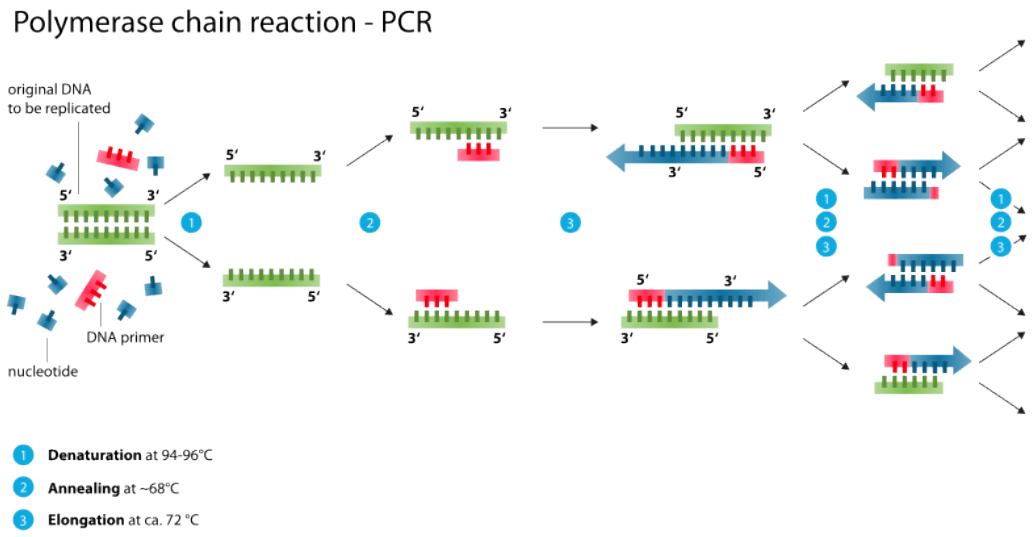


Figure 11.2.1: Schematic drawing of the Polymerase Chain Reaction (PCR) cycle. (CC-SA-BY-3.0; Enzoklop)

With PCR, it is routinely possible to amplify enough DNA from a single hair follicle for DNA typing. Some workers have successfully amplified DNA from a single sperm cell. The PCR technique has even made it possible to analyze DNA from microscope slides of tissue preserved years before. However, the great sensitivity of PCR makes contamination by extraneous DNA a constant problem.

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11.3: Gene Therapy - Methods and Prospects

Many human diseases are caused by defective genes. A few common examples are tabulate in Table 11.3.1; all of these diseases are caused by a defect at a **single gene locus**. (The inheritance is recessive so both the maternal and paternal copies of the gene must be defective.) Is there any hope of introducing functioning genes into these patients to correct their disorder? Probably. Other diseases also have a genetic basis, but it appears that several genes must act in concert to produce the disease phenotype. The prospects of gene therapy in these cases seems far more remote.

Table 11.3.1: Human Diseases cause by defective gene

Disease	Genetic defect
hemophilia A	absence of clotting factor VIII
cystic fibrosis	defective chloride channel protein
muscular dystrophy	defective muscle protein (dystrophin)
sickle-cell disease	defective beta globin
hemophilia B	absence of clotting factor IX
severe combined immunodeficiency (SCID)	any one of several genes fail to make a protein essential for T and B cell function

Severe Combined Immunodeficiency (SCID)

SCID is a disease in which the patient has neither cell-mediated immune responses nor is able to make antibodies. It is a disease of young children because, until recently, the absence of an immune system left them prey to infections that ultimately killed them. About 25% of the cases of SCID are the result of the child being homozygous for a defective gene encoding the enzyme **adenosine deaminase** (**ADA**). The normal catabolism of purines is deficient, and this is particularly toxic for **T cells** and B cells.

Treatment Options for SCID include:

1. Raise the child in a strictly **germfree environment**: all food, water, and air to be sterilized. David, the "bubble boy" from Houston, survived this way until he was 12 years old.
2. Give the child a transplant of **bone marrow** from a normal, histocompatible, donor. Ideally, this would give the child a continuous source of ADA^+ T and B cells. However, even though the child cannot reject the transplant (the child has no immune system), T cells in the transplant (unless the donor was an identical twin) can attack the cells of the child producing graft-versus-host disease. Moreover, the donor cells may be infected with a virus which could overwhelm the recipient before his or her immune system was restored. (David received a bone marrow transplant from his sister, but she, like many people, had been infected earlier with the **Epstein-Barr virus** (the cause of "mono")). The virus was still present in the cells she donated, and killed her brother.
3. Give **injections of ADA** (the enzyme is currently extracted from cows). When conjugated with polyethylene glycol (PEG) to delay its breakdown in the blood, ADA-PEG injections have kept SCID patients reasonably healthy. But just like the insulin injections of a diabetic, they must be repeated at frequent intervals. So,
4. Giving the patient functioning ADA genes - **gene therapy**

Gene Therapy Requirements

The gene must be identified and cloned. This has been done for the ADA gene. It must be inserted in cells that can take up long-term residence in the patient. So far, this means removing the **patient's own cells**, treating them in tissue culture, and then returning them to the patient. It must be inserted in the DNA so that it will be expressed adequately; that is, transcribed and translated with sufficient efficiency that worthwhile amounts of the enzyme are produced. All these requirements seem to have been met for SCID therapy using a **retrovirus** as the gene vector. Retroviruses have several advantages for introducing genes into human cells.

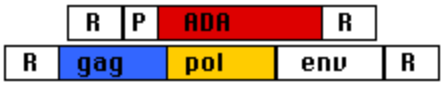


Figure 11.3.1: (left) ADA vector and (right) Retroviral Genome crippled

- Their envelope protein enables the virus to infect human cells.
- RNA copies of the human ADA gene can be incorporated into the retroviral genome using a packaging cell.

Packaging cells are treated so they express an RNA copy of the human ADA gene along with a **packaging signal** (**P**) needed for the assembly of fresh virus particles. They also needs **inverted repeats** ("**R**") at each end to aid insertion of the DNA copies into the DNA of the target cell. They need an RNA copy of the retroviral **gag**, **pol**, and **env** genes but with **no packaging signal** (so these genes cannot be incorporated in fresh viral particles).

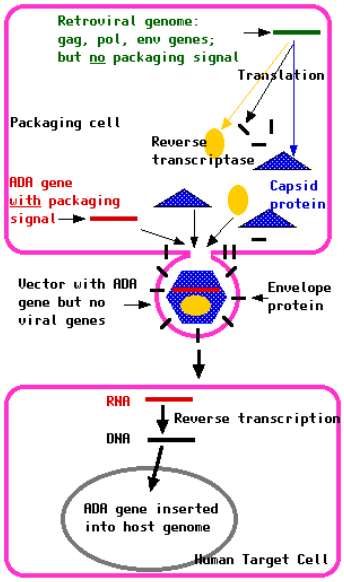


Figure 11.3.2: Packaging Cell

Treated with these two genomes, the packaging cell produces a crop of **retroviruses** with:

- the envelope protein needed to infect the **human target cells**
- an RNA copy of the human ADA gene, complete with R sequences at each end
- reverse transcriptase, needed to make a DNA copy of the ADA gene that can be inserted into the DNA of the target cell
- none of the genes (gag, pol, env) that would enable the virus to replicate in its new host.

Once the virus has infected the target cells, this RNA is reverse transcribed into DNA and inserted into the chromosomal DNA of the host.

Target Cells: T cells

The first attempts at gene therapy for SCID children (in 1990), used their own T cells (produced following ADA-PEG therapy) as the target cells. The T cells were:

- placed in tissue culture
- stimulated to proliferate (by treating them with the lymphokine, Interleukin 2 (IL-2)
- infected with the retroviral vector
- returned, in a series of treatments, to the child

The children developed improved immune function but the injections had to be repeated because T cells live for only 6–12 months in the blood. Moreover, the children also continued to receive ADA-PEG so the actual benefit of the gene therapy was unclear

Target Cells: Stem cells

Blood ("hematopoietic") stem cells:

- produce (by mitosis) all the types of blood cells, including T and B lymphocytes
- produce (by mitosis) **more stem cells**, thus ensuring an inexhaustible supply

In June of 2002, a team of Italian and Israeli doctors reported on two young SCID patients that were treated with their own blood stem cells that had been transformed in vitro with a retroviral vector carrying the ADA gene. After a year, both children had fully-functioning immune systems (T, B, and NK cells) and were able to live normal lives without any need for treatment with ADA-PEG or immune globulin (IG). The doctors attribute their success to first destroying some of the bone marrow cells of their patients to "make room" for the transformed cells. Nine years later (August 2011) these two patients are still thriving and have been joined by 28 other successfully-treated children most of whom no longer need to take ADA-PEG.

Gene Therapy for X-linked SCID

Gene therapy has also succeeded for 20 baby boys who suffered from another form of **severe combined immunodeficiency** called **X-linked SCID** because it is caused by a mutated X-linked gene encoding a subunit — called γc (gamma-c) — of the **receptor** for several interleukins, including **interleukin-7** (**IL-7**). IL-7 is essential for converting blood stem cells into the progenitors of **T cells**. Boys with X-linked SCID can make normal **B cells**, but because B cells need T-helper cells to function, these boys could make neither cell-mediated nor antibody-mediated immune responses and had to live in a sterile bubble before their treatment.

Their doctors

- isolated blood stem cells from the bone marrow of each infant
- treated the cells with a retroviral vector containing the normal gene for the γc interleukin receptor subunit
- returned the treated cells to each donor

The results: Now after as long as 11 years, 19 of these boys

- are able to live normal lives at home instead of inside a sterile "bubble"
- have normal (with some exceptions*) numbers of T cells of both the CD4 and CD8 subsets
- have responded to several childhood immunizations, including **diphtheria**, **tetanus** and **polio** by producing both T cells and antibodies specific for these agents
- Antibody production is sufficiently good that most of the boys have no need for periodic infusions of immune globulin (IG)

Five of the little boys developed leukemia (one has died):

- in one case caused by a proliferating clone of γδ T cells in which the vector has inserted itself in a gene (on chromosome 11) implicated in some cases of acute lymphoblastic leukemia (ALL)
- in a second case, the leukemia was of αβ T cells

Gene Therapy for β-thalassemia

β-thalassemia is an inherited disease. The most severe cases result from mutations in both copies of the gene encoding the beta chain of hemoglobin. Many causative mutations have been identified, and most lead to a failure to make any beta chains. The resulting hemoglobin functions poorly and the person requires frequent blood transfusions. In 2010, Cavazzana-Calvo (and many colleagues) report a single case of successful gene therapy for this disorder; their patient was an 18-year old male. Their procedure involve harvesting blood stem cells from the patient and exposing him to a retroviral vector that contained

- a human gene for beta-hemoglobin complete with its promoter, enhancer, and other control elements;
- alterations to the vector to make it safe.
- After sufficient chemotherapy to "make room" for them, the patient was injected with these cells.

The result: Almost three years later, the patient is well and no longer requires periodic blood transfusions. One-third of his hemoglobin is now manufactured by the red-cell precursors descended from the gene-altered **stem cells**. A similar procedure was used on several babies born with an inherited lysosomal storage disease or Wiskott-Aldrich syndrome (another type of immune deficiency). Up to two years after treatment with a retroviral vector containing the intact gene, these babies shown any signs of their disorders (reported in the 23 August 2013 issue of *Science*).

Adenovirus Vectors

Adenoviruses are human pathogens responsible for some cases of the human "cold". Modified versions of two strains are currently being used as vectors in gene therapy trials. Advantages of adenovirusus: Unlike retroviral vectors, they do not integrate into the host genome and thus should not be able to disrupt host genes (It was such disruption that caused some X-linked SCID patients treated with a retroviral vector to develop leukemia) and they can infect nondividing cells with high efficiency. Disadvantages are that they elicit a powerful immune response, both by T cells and by B cells (antibodies) so repeated doses soon lose their effectiveness. Moreoer, many people already have antibodies against the virus from earlier "colds", and these can inactivate the vector at the outset. A recent trial of an HIV vaccine using an adenovirus as the vector was halted when it was found not only not to be effective but, in people with preexisting high levels of anti-adenovirus antibodies, may have even increased their susceptibility to HIV.

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11.4: Recent Advances in Gene Therapy

Reaching the goal of effective gene therapies for human diseases has been a difficult one. Some of the problems that remain to be solved include:

- how to avoid an immune response in the patient, which can interfere with gene therapy in two ways:
  - The vector provokes inflammation.
  - The vector elicits antibodies that destroy the vector when it is administered again.

  Both of these are serious problems with adenovirus vectors.
- how to get the gene into non-dividing cells like liver, muscle, and neurons;
- how to get the gene to be replicated (in dividing cells) and expressed indefinitely but
- minimize the risk that it inserts near a proto-oncogene which it could activate producing a cancer. (This occurred in several little boys treated with a retroviral vector based on the murine leukemia virus.
- how to get the gene to be expressed **as needed**; that is, how to bring the gene under normal physiological controls so that its product is produced where, when, and in the amounts needed.

Adeno-Associated Virus (AAV) — A possible solution?

Adeno-associated virus gets its name because it is often found in cells that are simultaneously infected with adenovirus. However, by itself it seems to be harmless. Unlike adenovirus, AAV

- does not stimulate inflammation in the host;
- can enter non-dividing cells;
- integrates successfully into one spot in the genome of its host — on chromosome 19 in humans.
- (However, AAV vectors do elicit a strong immune response so they can be used only once.)

As for the problem of getting the transgene to be expressed appropriately, that may be solved by using **two** AAV vectors simultaneously:

- one carrying the desired gene (e.g., for factor VIII or adenosine deaminase, or in the case illustrated here, erythropoietin) into the cells of the host;
- the other carrying genes for the components of the transcription factors needed to turn that gene on.

Regulated production of erythropoietin (EPO)

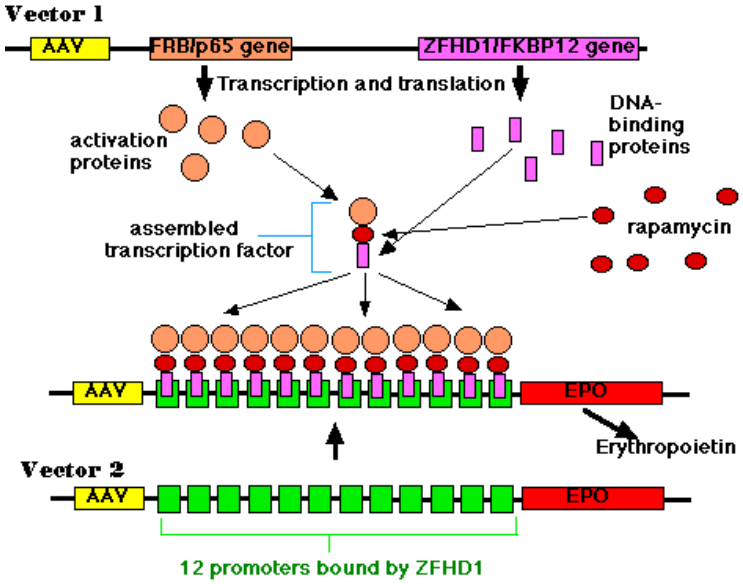


Figure 11.3.2.1 AAV Vectors

Vector 1

This piece of DNA contained (among other things):

- the DNA of adeno-associated virus (**AAV**)
- a gene encoding a protein containing two domains:
  - a portion of the molecule ("**p65**") that is needed to **activate gene transcription** but that by itself **cannot bind to DNA**
  - a portion ("**FRB**") that binds the drug **rapamycin**.
- a gene encoding another protein with two domains:
  - a portion of molecule ("**ZFHD1**") that binds specifically to the DNA sequence in the promoter of the erythropoietin gene but that by itself cannot activate transcription of the gene;
  - a portion ("**FKBP12**") that also binds to rapamycin.
- promoters (not shown) that allow continuous expression (transcription and translation) of the two genes. But note that, by themselves, the two gene products are inactive

Vector 2

This piece of DNA contained (among other things):

- the DNA of adeno-associated virus (**AAV**)
- 12 identical promoters (green boxes) of the erythropoietin gene
- the gene for erythropoietin (**EPO**) itself

The Experiment

The experimental animals were injected (in their skeletal muscles) with many copies of both vectors. Skeletal muscle was chosen because muscle fibers are multinucleate. Once across the plasma membrane, there are many nuclei which the vectors can enter and hence many opportunities to integrate into the DNA of the host.

Later the animals were injected with **rapamycin**. This small molecule is an immunosuppressant and is currently being tested in transplant recipients to help them avoid rejection of the transplant. It was used here because of its ability to simultaneously bind to the FRB and FKBP12 domains of the two gene products of vector 1. The resulting trimer is an active transcription factor for the erythropoietin gene.

The Results

In mice

Injections of the two vectors had — by themselves — no effect on the production of EPO nor on the number of red blood cells (hematocrit), but every time these animals were given an **injection of rapamycin**, they quickly began to produce **EPO** (with levels increasing as much as 100 fold) and the number of **red blood cells** rose (hematocrits increasing from 42% to 60%). The amount of EPO produced was directly related to the amount of rapamycin given. Even after 5 months, a single injection of rapamycin produced a sharp rise in the level of EPO in the blood.

In monkeys

The results were similar to those in mice, but the effect wore off after 4 months. So here is a system where a gene introduced into an animal can then be switched on by giving the animal a small molecule. (In humans, rapamycin can be given by mouth as a pill.) and can have its output regulated by the amount of the small molecule administered.

Curing Insulin-Dependent Diabetes Mellitus (IDDM) in mice and rats

Researchers in Seoul, Korea reported in the 23 November 2000 issue of **Nature** that they have used an AAV-type vector to cure

- mice with inherited IDDM (the animal equivalent of Type 1 diabetes mellitus in humans)

- rats with IDDM induced by chemical destruction of their insulin-secreting beta cells

Both groups of animals were injected (in their hepatic portal vein) with billions of copies of a complex vector containing:

- **AAV**
- the complementary DNA (cDNA) encoding a synthetic version of insulin
- a promoter that is active only in liver cells and is **turned on by the presence of glucose**
- the DNA encoding a signal sequence (so that the insulin can be secreted)
- an enhancer to elevate expression of this artificial gene

The results:

Both groups of animals gained control over their blood sugar level and kept this control for over 8 months. When given glucose, they proceeded to synthesize the synthetic insulin which then brought their blood glucose back down to normal levels.

Curing hemophilia B in mice

Researchers at the Salk Institute reported (in the 30 March 1999 issue of the **Proceedings of the National Academy of Sciences**) work with mice

- whose genes for clotting factor IX had been "knocked out" and
- thus were subject to uncontrolled bleeding like human patients with hemophilia B.

These mice were injected (also in the hepatic portal vein) with DNA containing

- AAV
- cDNA for factor IX (the dog gene)
- liver-specific promoter and enhancer sequences

The mice proceeded to make factor IX and were no longer susceptible to uncontrolled bleeding.

In later work, injection of embryonic stem cells with functioning factor IX genes into the liver of mice without the genes cured them.

Treating ALS

ALS (**a**myotrophic **l**ateral **s**clerosis) is a human disease in which motor neurons degenerate. (It is often called "Lou Gehrig's disease" after the baseball player who died from it.)

A similar disease can be created in transgenic mice carrying mutant human genes (for superoxide dismutase) associated with ALS.

Researchers at the Salk Institute have slowed up the progression of the disease in these mice by injecting their skeletal muscles with an AAV vector containing the gene for insulin-like growth factor 1 (IGF-1). The vector

- invaded the muscle cells
- moved into the motor neurons attached to them and
- through their axons up to the cell bodies

The results:

Destruction of motor neurons was reduced, and the mice lived longer than they otherwise would have.

The Outlook

It's a big jump from mice to humans, but these results indicate that the principle of gene therapy for single-gene disorders is valid.

And some early trials in humans look promising.

- An intravenous injection of an AAV vector containing the cDNA of factor IX has produced functional levels of factor IX in several men with **hemophilia B**.
- On August 18, 2003, physicians in New York injected 3.5 x 10^9 copies of an AAV vector carrying a gene for the synthesis of GABA into the brain of a patient with Parkinson's disease. He was the first of a phase I clinical trial of this procedure. By 2007, several more Parkinson's patients had been treated with these injections with no harmful side effects and some improvement in their symptoms.
- Several patients with an inherited lack of a functional gene needed to synthesize 11-*cis*-retinal — and thus destined to be blind — have had a useful level of vision temporarily restored in one eye injected with an AAV vector containing the gene (the other eye was the untreated control). Probably the fact that
  - the vector was injected directly into the eye and so not diluted throughout the body as an intravenous injection would be;
  - retinal cells rarely divide so the vector would not be lost. (The vector used had the genes needed for integration into the host cell's DNA removed so it could not be duplicated in S phase and, in dividing cells, would eventually disappear.)
  - the interior of the eye is an immunologically privileged site

  contributed to this remarkable success.
- Several children suffering from X-linked severe combined immunodeficiency have had their immune systems restored after **retroviral** gene therapy.
- A few patients with hemophilia A have shown modest improvement when injected with their own cells that had earlier been harvested and transformed in vitro with a plasmid containing the factor VIII gene.
- Several gene therapy agents — using adenoviral vectors — are in clinical trials and have shown some promise.

  Among these:

  - a recombinant adenovirus encoding p53, a tumor-suppressor protein missing in many cancers
  - a recombinant adenovirus that destroys cells lacking the p53 protein (as many cancer cells do)

People with the rare disorder **lipoprotein lipase deficiency** are unable to process the globules (chylomicrons) of fat and protein that appear in the blood after a fat-containing meal because they lack functional copies of the gene encoding lipoprotein lipase. Intramuscular injection of an AAV vector containing the functional gene provides sufficient improvement, with apparent safety, that in October 2012, this agent (Glybera®) received approval for use in the European Union. It is the first gene therapy to receive such approval.

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11.5: Transgenic Animals

A transgenic animal is one that carries a foreign gene that has been deliberately inserted into its genome. The foreign gene is constructed using recombinant DNA methodology. In addition to the gene itself, the DNA usually includes other sequences to enable it to be incorporated into the DNA of the host and to be expressed correctly by the cells of the host. Transgenic sheep and goats have been produced that express foreign proteins in their milk. Transgenic chickens are now able to synthesize human proteins in the "white" of their eggs. These animals should eventually prove to be valuable sources of proteins for human therapy.

Note

In July 2000, researchers from the team that produced Dolly reported success in producing transgenic lambs in which the transgene had been inserted at a specific site in the genome and functioned well.

Transgenic **mice** have provided the tools for exploring many biological questions.

Example

Normal mice cannot be infected with polio virus. They lack the cell-surface molecule that, in humans, serves as the receptor for the virus. So normal mice cannot serve as an inexpensive, easily-manipulated model for studying the disease. However, transgenic mice expressing the human gene for the polio virus receptor

- can be infected by polio virus and even
- develop paralysis and other pathological changes characteristic of the disease in humans.

Two methods of producing transgenic mice are widely used:

- transforming **embryonic stem cells** (**ES** cells) growing in tissue culture with the desired DNA
- injecting the desired gene into the **pronucleus** of a fertilized mouse egg

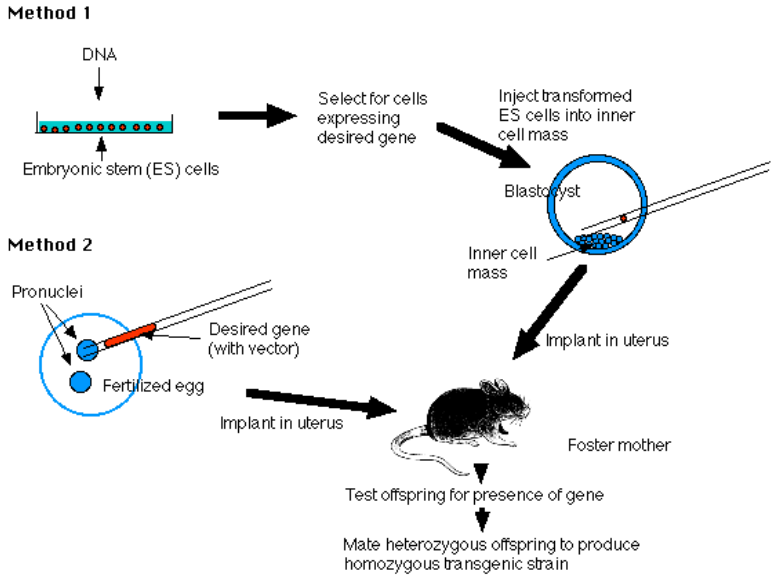


Fig.11.4.1 Methods to produce Transgenic mice

The Embryonic Stem Cell Method - Method 1

Embryonic stem cells (**ES** cells) are harvested from the **inner cell mass** (ICM) of mouse blastocysts. They can be grown in culture and retain their full potential to produce all the cells of the mature animal, **including its gametes**.

1. Make your DNA

Using recombinant DNA methods, build molecules of DNA containing

- the gene you desire (e.g., the insulin gene)
- **vector** DNA to enable the molecules to be inserted into host DNA molecules
- **promoter and enhancer sequences** to enable the gene to be expressed by host cells

2. Transform ES cells in culture

Expose the cultured cells to the DNA so that some will incorporate it.

3. Select for successfully transformed cells

4. Inject these cells into the inner cell mass (ICM) of mouse blastocysts.

5. Embryo transfer

- Prepare a **pseudopregnant** mouse (by mating a female mouse with a vasectomized male). The stimulus of mating elicits the hormonal changes needed to make her uterus receptive.
- Transfer the embryos into her uterus.
- Hope that they **implant** successfully and develop into healthy pups (no more than one-third will).

6. Test her offspring

- Remove a small piece of tissue from the tail and examine its DNA for the desired gene. No more than 10–20% will have it, and they will be heterozygous for the gene.

7. Establish a transgenic strain

- Mate two heterozygous mice and screen their offspring for the 1 in 4 that will be **homozygous** for the transgene.
- Mating these will found the transgenic strain.

The Pronucleus Method - Method 2

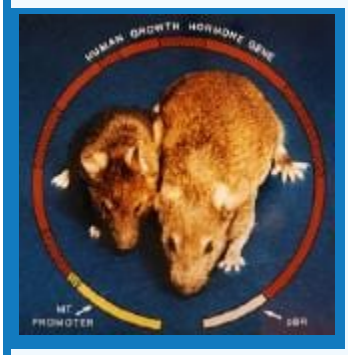
1. Prepare your DNA as in Method 1

2. Transform fertilized eggs

- Harvest freshly fertilized eggs before the sperm head has become a pronucleus.
- Inject the male pronucleus with your DNA.
- When the pronuclei have fused to form the diploid zygote nucleus, allow the zygote to divide by mitosis to form a 2-cell embryo.

3. Implant the embryos in a pseudopregnant foster mother and proceed as in Method 1.

Example



This image (courtesy of R. L. Brinster and R. E. Hammer) shows a transgenic mouse (right) with a normal littermate (left). The giant mouse developed from a fertilized egg transformed with a recombinant DNA molecule containing:

- the gene for **human growth hormone**
- a strong mouse gene **promoter**

The levels of growth hormone in the serum of some of the transgenic mice were several hundred times higher than in control mice.

Random vs. Targeted Gene Insertion

The early vectors used for gene insertion could, and did, place the gene (from one to 200 copies of it) anywhere in the genome. However, if you know some of the DNA sequence flanking a particular gene, it is possible to design vectors that replace that gene. The replacement gene can be one that

- restores function in a mutant animal or
- knocks out the function of a particular locus.

In either case, targeted gene insertion requires

- the desired gene
- ***neo*^{r}**, a gene that encodes an enzyme that inactivates the antibiotic neomycin and its relatives, like the drug G418, which is lethal to mammalian cells
- ***tk***, a gene that encodes **thymidine kinase**, an enzyme that phosphorylates the nucleoside analog **ganciclovir**. **DNA polymerase** fails to discriminate against the resulting nucleotide and inserts this nonfunctional nucleotide into freshly-replicating DNA. So ganciclovir kills cells that contain the *tk* gene

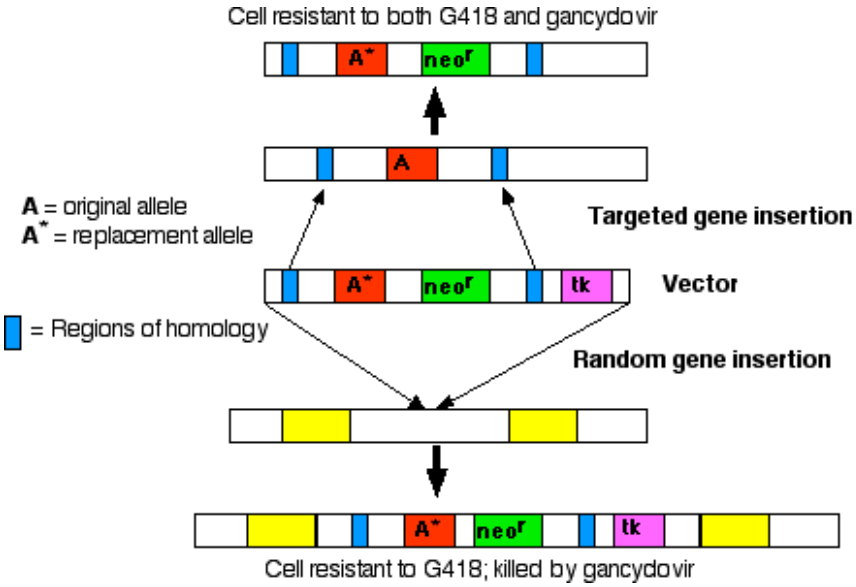


Figure 11.4.2 Neo vector

Step 1

Treat culture of ES cells with preparation of vector DNA.

Results:

- **Most cells** fail to take up the vector; these cells will be killed if exposed to G418.
- In a **few cells**: the vector is inserted randomly in the genome. In random insertion, the entire vector, including the ***tk*** gene, is inserted into host DNA. These cells are resistant to G418 but killed by gancyclovir.
- In **still fewer cells**: **homologous recombination** occurs. Stretches of DNA sequence in the vector find the homologous sequences in the host genome, and the region between these homologous sequences replaces the equivalent region in the host DNA.

Step 2

Culture the mixture of cells in medium containing both G418 and ganciclovir.

- The cells (the majority) that failed to take up the vector are killed by G418.
- The cells in which the vector was inserted randomly are killed by gancyclovir (because they contain the *tk* gene).
- This leaves a population of cells transformed by homologous recombination (enriched several thousand fold).

Step 3

Inject these into the inner cell mass of mouse blastocysts.

Knockout Mice: What do they teach us?

If the replacement gene (**A*** in the diagram) is nonfunctional (a "null" allele), mating of the heterozygous transgenic mice will produce a strain of "**knockout mice**" homozygous for the nonfunctional gene (both copies of the gene at that locus have been "knocked out"). Knockout mice are valuable tools for discovering the function(s) of genes for which mutant strains were not previously available. Two generalizations have emerged from examining knockout mice:

- Knockout mice are often surprisingly unaffected by their deficiency. Many genes turn out not to be indispensable. The mouse genome appears to have sufficient redundancy to compensate for a single missing pair of alleles.
- Most genes are **pleiotropic**. They are expressed in different tissues in different ways and at different times in development.

Tissue-Specific Knockout Mice

While "housekeeping" genes are expressed in all types of cells at all stages of development, other genes are normally expressed in only certain types of cells when turned on by the appropriate signals (e.g. the arrival of a hormone).

To study such genes, one might expect that the methods described above would work. However, it turns out that genes that are only expressed in certain adult tissues may nonetheless be vital during embryonic development. In such cases, the animals do not survive long enough for their knockout gene to be studied. Fortunately, there are now techniques with which transgenic mice can be made where a particular gene gets knocked out in only one type of cell.

The Cre/*loxP* System

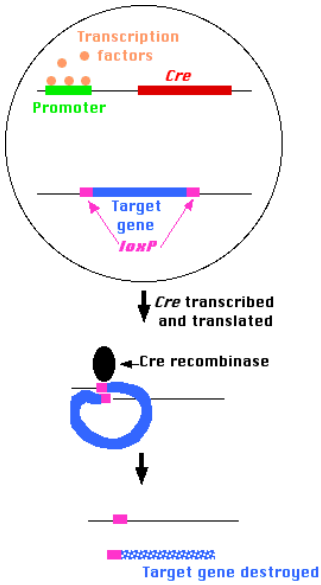


Figure 11.4.3 The Cre/loxP System

One of the bacteriophages that infects E. coli, called P1, produces an enzyme — designated Cre — that cuts its DNA into lengths suitable for packaging into fresh virus particles. Cre cuts the viral DNA wherever it encounters a pair of sequences designated *loxP*. All the DNA between the two *loxP* sites is removed, and the remaining DNA ligated together again (so the enzyme is a recombinase). Using "Method 1" above, mice can be made transgenic for

- the gene encoding Cre attached to a promoter that will be activated only when it is bound by the same transcription factors that turn on the other genes required for the unique function(s) of that type of cell;
- a "target" gene, the one whose function is to be studied, flanked by *loxP* sequences.

In the adult animal,

- those cells that
  - receive signals (e.g., the arrival of a hormone or cytokine)
  - to turn on production of the transcription factors needed
  - to activate the promoters of the genes whose products are needed by that particular kind of cell

  will also turn on transcription of the Cre gene. Its protein will then remove the "target" gene under study.
- All other cells will lack the transcription factors needed to bind to the Cre promoter (and/or any enhancers) so the target gene remains intact.

The result: a mouse with a particular gene knocked out in only certain cells.

Knock-in Mice

The Cre/*loxP* system can also be used to

- remove DNA sequences that block gene transcription. The "target" gene can then be turned **on** in certain cells or at certain times as the experimenter wishes.
- replace one of the mouse's own genes with a new gene that the investigator wishes to study.

Such transgenic mice are called "knock-in" mice.

Transgenic Sheep and Goats

Until recently, the transgenes introduced into sheep inserted randomly in the genome and often worked poorly. However, in July 2000, success at inserting a transgene into a specific gene locus was reported. The gene was the human gene for **alpha1-antitrypsin**, and two of the animals expressed large quantities of the human protein in their milk.

This is how it was done.

Sheep fibroblasts (connective tissue cells) growing in tissue culture were treated with a vector that contained these segments of DNA:

1. 2 regions homologous to the sheep *COL1A1* gene. This gene encodes Type 1 collagen. (Its absence in humans causes the inherited disease osteogenesis imperfecta.)

   This locus was chosen because fibroblasts secrete large amounts of collagen and thus one would expect the gene to be easily accessible in the chromatin.

2. A neomycin-resistance gene to aid in isolating those cells that successfully incorporated the vector.
3. The human gene encoding alpha1-antitrypsin.

   Some people inherit two non- or poorly-functioning genes for this protein. Its resulting low level or absence produces the disease **Alpha1-Antitrypsin Deficiency** (**A1AD** or **Alpha1**). The main symptoms are damage to the lungs (and sometimes to the liver).

4. Promoter sites from the **beta-lactoglobulin** gene. These promote hormone-driven gene expression in milk-producing cells.
5. Binding sites for ribosomes for efficient translation of the beta-lactoglobulin mRNAs.

Successfully-transformed cells were then

- Fused with enucleated sheep eggs and implanted in the uterus of a ewe (female sheep)
- Several embryos survived until their birth, and two young lambs lived over a year.
- When treated with hormones, these two lambs secreted milk containing large amounts of alpha1-antitrypsin (650 µg/ml; 50 times higher than previous results using random insertion of the transgene).

On June 18, 2003, the company doing this work abandoned it because of the great expense of building a facility for purifying the protein from sheep's milk. Purification is important because even when 99.9% pure, human patients can develop antibodies against the tiny amounts of sheep proteins that remain.

However, another company, GTC Biotherapeutics, has persevered and in June of 2006 won preliminary approval to market a human protein, antithrombin, in Europe. Their protein — the first made in a transgenic animal to receive regulatory approval for human therapy — was secreted in the milk of transgenic goats.

Transgenic Chickens

Chickens grow faster than sheep and goats and large numbers can be grown in close quarters. They also synthesize several grams of protein in the "white" of their eggs.Two methods have succeeded in producing chickens carrying and expressing foreign genes.

- Infecting embryos with a viral vector carrying
  - the human gene for a therapeutic protein
  - promoter sequences that will respond to the signals for making proteins (e.g. lysozyme) in egg white
- Transforming rooster sperm with a human gene and the appropriate promoters and checking for any transgenic offspring.

Preliminary results from both methods indicate that it may be possible for chickens to produce as much as 0.1 g of human protein in each egg that they lay.

Not only should this cost less than producing therapeutic proteins in culture vessels, but chickens will probably add the correct sugars to glycosylated proteins — something that E. coli cannot do.

Transgenic Pigs

Transgenic pigs have also been produced by fertilizing normal eggs with sperm cells that have incorporated foreign DNA. This procedure, called sperm-mediated gene transfer (SMGT) may someday be able to produce transgenic pigs that can serve as a source of transplanted organs for humans.

Transgenic Primates

In the 28 May 2009 issue of **Nature**, Japanese scientists reported success in creating transgenic marmosets. Marmosets are primates and thus our closest relatives (so far) to be genetically engineered. In some cases, the transgene (for green fluorescent protein) was incorporated into the germline and passed on to the animal's offspring. The hope is that these transgenic animals will provide the best model yet for studying human disease and possible therapies.

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11.6: Transgenic Plants

Progress is being made on several fronts to introduce new traits into plants using recombinant DNA technology. The genetic manipulation of plants has been going on since the dawn of agriculture, but until recently this has required the slow and tedious process of cross-breeding varieties. Genetic engineering promises to speed the process and broaden the scope of what can be done.

There are several methods for introducing genes into plants, including infecting plant cells with plasmids as vectors carrying the desired gene and physically shooting microscopic pellets containing the gene directly into the cell. In contrast to animals, there is no real distinction between somatic cells and germline cells. Somatic tissues of plants (e.g., root cells grown in culture) can be transformed in the laboratory with the desired gene and can grow into mature plants with flowers. If all goes well, the transgene will be incorporated into the pollen and eggs and passed on to the next generation. In this respect, it is easier to produce transgenic plants than transgenic animals.

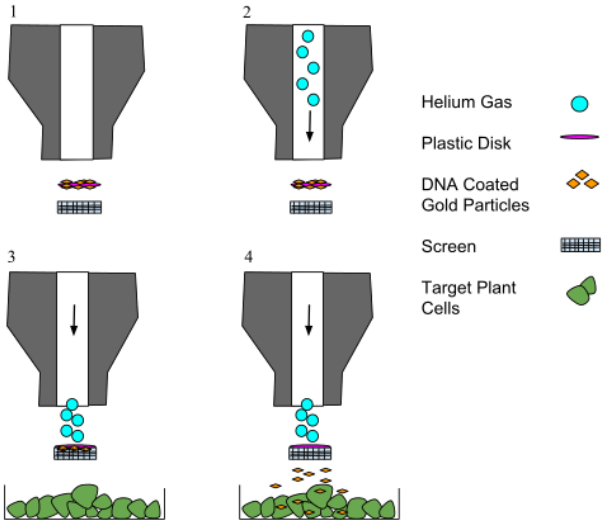


Figure 11.6.1: A gene gun is used for injecting cells with genetic information, it is also known as biolistic particle delivery system. Gene guns can be used effectively on most cells but are mainly used on plant cells. Step 1 The gene gun apparatus is ready to fire. Step 2 When the gun is turned on and the helium flows through. Step 3 The helium moving the disk with DNA coated particles toward the screen. Step 4 The helium having pushed the particles moving through the screen and moving to the target cells to transform the cells. (CC-SA-BY-4.0; RachelBrooks15)

Achievements

Improved Nutritional Quality

Milled rice is the staple food for a large fraction of the world's human population. Milling rice removes the husk and any beta-carotene it contained. Beta-carotene is a precursor to vitamin A, so it is not surprising that vitamin A deficiency is widespread, especially in the countries of Southeast Asia. The synthesis of beta-carotene requires a number of enzyme-catalyzed steps. In January 2000, a group of European researchers reported that they had succeeded in incorporating **three transgenes** into rice that enabled the plants to manufacture beta-carotene in their endosperm.

Insect Resistance

Bacillus thuringiensis is a bacterium that is pathogenic for a number of insect pests. Its lethal effect is mediated by a protein toxin it produces. Through recombinant DNA methods, the toxin gene can be introduced directly into the genome of the plant where it is expressed and provides protection against insect pests of the plant.

Disease Resistance

Genes that provide resistance against plant viruses have been successfully introduced into such crop plants as tobacco, tomatoes, and potatoes.

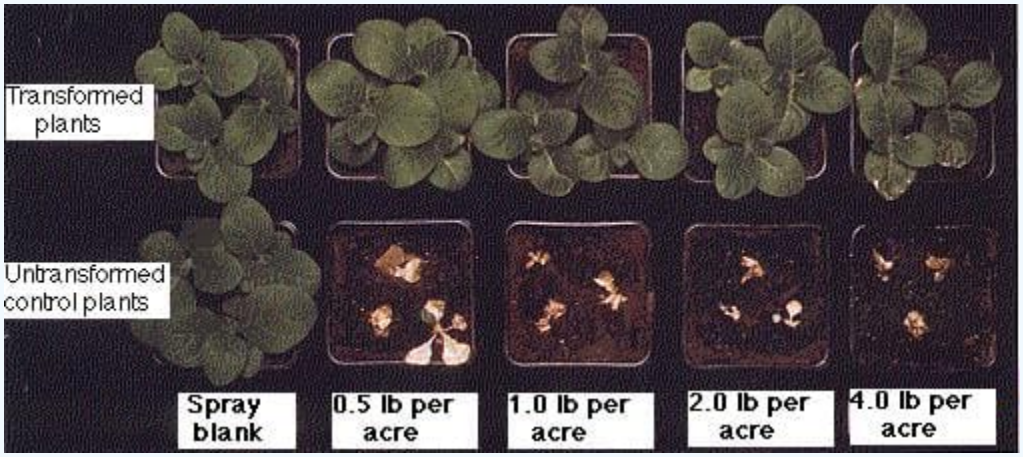
Example 11.6.1

Tomato plants infected with **tobacco mosaic virus** (which attacks tomato plants as well as tobacco). The plants in the back row carry an introduced gene conferring resistance to the virus. The resistant plants produced three times as much fruit as the sensitive plants and the same as control plants.

Herbicide Resistance

Questions have been raised about the safety — both to humans and to the environment — of some of the broad-leaved weed killers like 2,4-D. Alternatives are available, but they may damage the crop as well as the weeds growing in it. However, genes for resistance to some of the newer herbicides have been introduced into some crop plants and enable them to thrive even when exposed to the weed killer.

Example 11.6.2



Effect of the herbicide bromoxynil on tobacco plants transformed with a bacterial gene whose product breaks down bromoxynil (top row) and control plants (bottom row). "Spray blank" plants were treated with the same spray mixture as the others except the bromoxynil was left out. (Courtesy of Calgene, Davis, CA.)

Salt Tolerance

A large fraction of the world's irrigated crop land is so laden with salt that it cannot be used to grow most important crops. However, researchers at the University of California Davis campus have created transgenic tomatoes that grow well in saline soils. The transgene was a highly-expressed sodium/proton antiport pump that sequestered excess sodium in the vacuole of leaf cells. There was no sodium buildup in the fruit.

"Terminator" Genes

This term is used (by opponents of the practice) for transgenes introduced into crop plants to make them produce sterile seeds (and thus force the farmer to buy fresh seeds for the following season rather than saving seeds from the current crop). The process involves introducing three transgenes into the plant:

- A gene encoding a **toxin** which is lethal to developing seeds but not to mature seeds or the plant. This gene is normally inactive because of a stretch of DNA inserted between it and its promoter.
- A gene encoding a **recombinase** — an enzyme that can remove the spacer in the toxin gene thus allowing to be expressed.
- A **repressor** gene whose protein product binds to the promoter of the recombinase thus keeping it inactive.

How they work

When the seeds are soaked (before their sale) in a solution of tetracycline

- Synthesis of the repressor is blocked.
- The recombinase gene becomes active.
- The spacer is removed from the toxin gene and it can now be turned on.

Because the toxin does not harm the growing plant — only its developing seeds — the crop can be grown normally except that its seeds are sterile. The use of terminator genes has created much controversy. Farmers — especially those in developing countries — want to be able to save some seed from their crop to plant the next season. However, Seed companies want to be able to keep selling seeds.

Transgenes Encoding Antisense RNA

Messenger RNA (mRNA) is single-stranded. Its sequence of nucleotides is called "sense" because it results in a gene product (protein). Normally, its unpaired nucleotides are "read" by transfer RNA anticodons as the ribosome proceeds to **translate** the message.

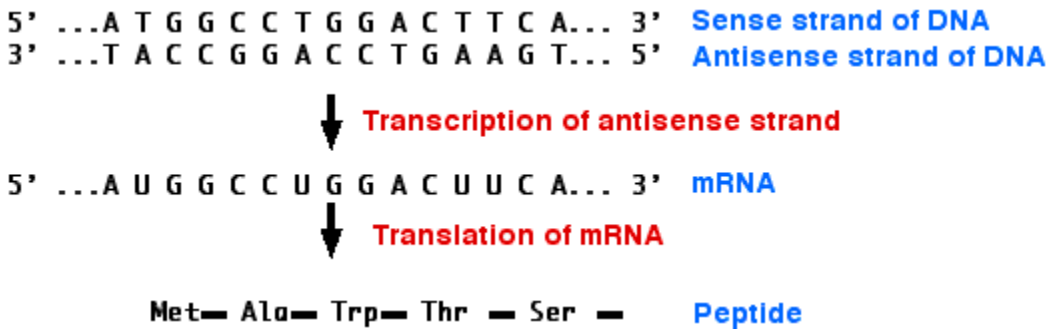


Figure 11.6.2: Sense Strand

The second strand is called the **antisense strand** because its sequence of nucleotides is the complement of message sense. When mRNA forms a duplex with a complementary antisense RNA sequence, translation is blocked. This may occur because the ribosome cannot gain access to the nucleotides in the mRNA or duplex RNA is quickly degraded by ribonucleases in the cell. With recombinant DNA methods, synthetic genes (DNA) encoding antisense RNA molecules can be introduced into the organism.

Biopharmaceuticals

The genes for proteins to be used in human (and animal) medicine can be inserted into plants and expressed by them.

Advantages:

- Glycoproteins can be made (bacteria like E. coli cannot do this).
- Virtually unlimited amounts can be grown in the field rather than in expensive fermentation tanks.
- It avoids the danger from using mammalian cells and tissue culture medium that might be contaminated with infectious agents.
- Purification is often easier.

Corn is the most popular plant for these purposes, but tobacco, tomatoes, potatoes, rice and carrot cells grown in tissue culture are also being used.

Some of the proteins that have been produced by transgenic crop plants:

- human growth hormone with the gene inserted into the chloroplast DNA of tobacco plants
- humanized antibodies against such infectious agents as
  - HIV
  - respiratory syncytial virus (RSV)
  - sperm (a possible contraceptive)
  - herpes simplex virus, HSV, the cause of "cold sores"
  - Ebola virus, the cause of the often-fatal Ebola hemorrhagic fever
- protein antigens to be used in vaccines
  - An example: patient-specific antilymphoma (a cancer) vaccines. B-cell lymphomas are clones of malignant B cells expressing on their surface a unique antibody molecule. Making tobacco plants transgenic for the RNA of the variable (unique) regions of this antibody enables them to produce the corresponding protein. This can then be incorporated into a vaccine in the hopes (early trials look promising) of boosting the patient's immune system — especially the cell-mediated branch — to combat the cancer.
- other useful proteins like lysozyme and trypsin
- However, as of April 2012, the only protein to receive approval for human use is glucocerebrosidase, an enzyme lacking in Gaucher's disease. It is synthesized by transgenic carrot cells grown in tissue culture.

Controversies

The introduction of transgenic plants into agriculture has been vigorously opposed by some. There are a number of issues that worry the opponents. One of them is the potential risk of transgenes in commercial crops endangering native or nontarget species.

Examples:

- A gene for herbicide resistance in, e.g. maize (corn), escaping into a weed species could make control of the weed far more difficult.
- The gene for Bt toxin expressed in pollen might endanger pollinators like honeybees.

To date, field studies on Bt cotton and maize show that the numbers of some nontarget insects are reduced somewhat but not as much as in fields treated with insecticides.

Another worry is the inadvertent mixing of transgenic crops with nontransgenic food crops. Although this has occurred periodically, there is absolutely no evidence of a threat to human health. Despite the controversies, farmers around the world are embracing transgenic crops. Currently in the United States over 80% of the corn, soybeans, and cotton grown are genetically modified (GM) — principally to provide resistance to the herbicide glyphosate ("Roundup Ready®") thus making it practical to spray the crop with glyphosate to kill weeds without harming the crop and resistance to insect attack (by expressing the toxin of *Bacillus thuringiensis*).

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11.7: Restriction Fragment Length Polymorphisms

Restriction enzymes cut DNA at precise points producing a collection of DNA fragments of precisely defined length. These can be separated by electrophoresis, with the smaller fragments migrating farther than the larger fragments. One or more of the fragments can be visualized with a "probe" — a molecule of single-stranded DNA that is complementary to a run of nucleotides in one or more of the restriction fragments and is radioactive (or fluorescent). If probes encounter a complementary sequence of nucleotides in a test sample of DNA, they bind to it by Watson-Crick base pairing and thus identify it. Polymorphisms are inherited differences found among the individuals in a population.

Restriction Fragment Length Polymorphisms (RFLPs) have provided valuable information in many areas of biology, including screening human DNA for the presence of potentially deleterious genes ("Case 1") and providing evidence to establish the innocence of, or a probability of the guilt of, a crime suspect by DNA "fingerprinting" ("Case 3").

Case 1: Screening for the sickle-cell gene

Sickle-cell disease is a genetic disorder in which both genes in the patient encode the amino acid **valine** (Val) in the sixth position of the beta chain (beta^{S}) of the hemoglobin molecule. "Normal" beta chains (beta^{A}) have **glutamic acid** at this position. The only difference between the two genes is the substitution of a T for an A in the middle position of codon 6. This converts a GAG codon (for Glu) to a GTG codon for Val and abolishes a sequence (CTGAGG, which spans codons 5, 6, and 7) recognized and cut by one of the restriction enzymes.

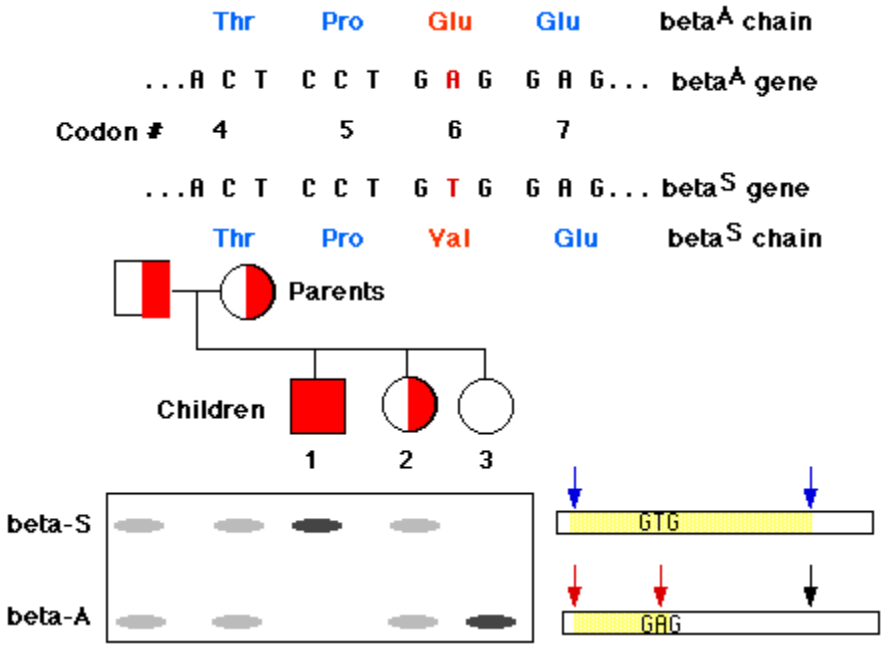


Figure 11.7.1: Sickle-cell Mutation. Data provided by S. E. Antonarakis

When the **normal** gene (beta^{A}) is digested with the enzyme and the fragments separated by electrophoresis, the probe binds to a **short** fragment (between the red arrows). However, the enzyme cannot cut the **sickle-cell gene** at this site, so the probe attaches to a much larger fragment (between the blue arrows).

Figure 11.7.1 shows the pedigree of a family whose only son has sickle-cell disease. Both his father and mother were heterozygous (semifilled box and circle respectively) as they had to be to produce an afflicted child (solid box). The electrophoresis patterns for each member of the family are placed directly beneath them. Note that the two homozygous children (1 and 3) have only a single band, but these are more intense because there is twice as much DNA in them. In this example, a change of a single nucleotide produced the RFLP. This is a very common cause of RFLPs and now such polymorphisms are often referred to as

single nucleotide polymorphisms or **SNPs**. (However, not all RFLPs arise from SNPs.

How can these tools be used?

By testing the DNA of prospective parents, their genotype can be determined and their odds of producing an afflicted child can be determined. In the case of sickle-cell disease, if **both** parents are heterozygous for the genes, there is a 1 in 4 chance that they will produce a child with the disease. Amniocentesis and chorionic villus sampling make it possible to apply the same techniques to the DNA of a fetus early in pregnancy. The parents can learn whether the unborn child will be free of the disease or not. They may choose to have an abortion rather than bring an afflicted child into the world.

Three problems:

- The mutations that cause most human genetic diseases are more varied than the single mutation associated with sickle-cell disease. Over a thousand different mutations in the cystic fibrosis gene can cause the disease. A probe for one will probably fail to identify a second. A mixture of probes, one for each of the more common mutations, can be used. But there remains the problem of "false negatives": people who are falsely told they do not carry a mutant gene.
- There are many diseases which result from several mutant genes working together to produce the disease phenotype.
- There are still genetic diseases for which no gene has yet been discovered. Until the gene can be located, cloned, and sequenced, no probe can be made to detect it directly. However, it is sometimes possible to find a genetic "marker" that can serve as a surrogate for the gene itself. Let's see how.

Case 2: Screening for a RFLP "marker"

If a particular RFLP is usually associated with a particular genetic disease, then the presence or absence of that RFLP can be used to counsel people about their risk of developing or transmitting the disease. The assumption is that the gene they are really interested in is located so close to the RFLP that the presence of the RFLP can serve as a surrogate for the disease gene itself. But people wanting to be tested cannot simply walk in off the street. Because of crossing over, a particular RFLP might be associated with the mutant gene in some people, with its healthy allele in others. Thus it is essential to examine not only the patient but as many members of the patient's family as possible.

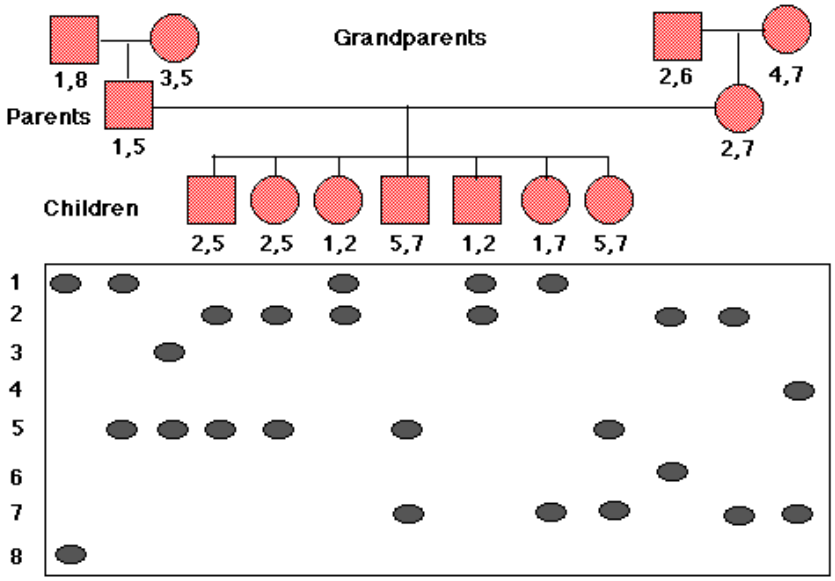


Figure 11.7.2: RFLP Marker

The most useful probes for such analysis bind to a unique sequence of DNA; that is, a sequence occurring at only one place in the genome. Often this DNA is of unknown, if any, function. This can actually be helpful as this DNA has been freer to mutate without harm to the owner. The probe will hybridize (bind to) different lengths of digested DNA in different people depending on where the enzyme cutting sites are that each person has inherited. Thus a large variety of alleles (polymorphisms) may be present in the population. Some people will be homozygous and reveal a single band; others (e.g., all the family members shown below) will be

heterozygous with each allele producing its band.

The pedigree in Figure 11.7.2 shows the inheritance of a RFLP marker through three generations in a single family. A total of 8 alleles (numbered to the left of the blots) are present in the family. The RFLPs of each member of the family are placed directly below his (squares) or her (circles) symbol and RFLP numbers.

If, for example, everyone who inherited RFLP 2 also has a certain inherited disorder, and no one lacking RFLP 2 has the disorder, we deduce that the gene for the disease is closely linked to this RFLP. If the parents decide to have another child, prenatal testing could reveal whether that child was apt to come down with the disease. But note, that crossing over during gamete formation could have moved the RFLP to the healthy allele. So the greater the distance between the RFLP and the gene locus, the lower the probability of an accurate diagnosis.

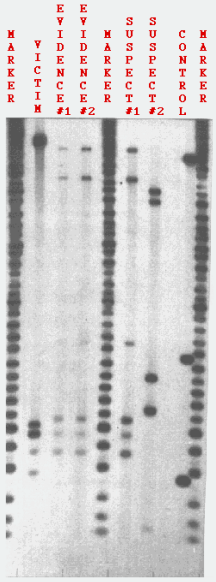
Case 3: DNA "typing"

Each human cell contains 6 x 10^9 base pairs of DNA. Some of this represents protein-encoding genes (e.g., for the beta chain of hemoglobin) that are identical in a large proportion of people. But long stretches of DNA do not encode for anything and are free to mutate extensively. It seems certain that if we could read the entire sequence of DNA in each human, we would never find two that were identical (unless the samples were from identical siblings; i.e., derived from a single zygote).

So each person's DNA is as unique as a fingerprint. This truth has not escaped the law enforcement and legal professions. Analysis of DNA, called **DNA typing**, is widely used to identify rapists and other criminals, determine paternity; that is, who the father of the child really is, determine whether a hopeful immigrant is, as he or she claims, really a close relative of already established residents.

Example 11.7.1: Rape Suspect

Figure 11.7.3 shows the test results in a rape case. Two probes were used: one revealing the bands at the top, the other those at the bottom.



3

Figure 11.7.3: DNA typing courtesy of Lifecodes Corporation

DNA was tested from

- semen removed from the vagina of the rape victim (EVIDENCE #2);
- a semen stain left on the victim's clothing (EVIDENCE #1);
- the DNA of the victim herself (VICTIM) to be sure that the DNA didn't come from her cells;

- DNA from two suspects (SUSPECT #1, SUSPECT #2);
- a set of DNA fragments of known and decreasing length (MARKER). They provide a built-in ruler for measuring the exact distance that each fragment travels.
- the cells of a previously-tested person to be sure the probes are performing properly (CONTROL).

One the basis of this test, **suspect #2** can clearly be **ruled out**. None of his bands matches the bands found in the semen.

Is **suspect #1** guilty?

We can never be certain. The best we can do is to estimate the probability that another person, picked at random, could provide the same DNA fingerprint. As a conservative estimate, a given allele (band) might be found in 25% of the people tested. The probability of a random match of two alleles is $(0.25)^2$ or 1 in 16. The probability that 6 alleles match, as in this case, is $(0.25)^6$ or 1 in 4096. However, the suspect was not picked at random, so you may feel that the evidence of guilt is strong.

The more probes you use, the more confident you can be that you have gotten the right man. If, for example, a set of probes revealed 14 bands in a suspect's DNA identical to those in the semen sample, the probability that you have the wrong man drops to less than 1 in 268 million $(0.25)^{14}$ = 1/268,435,456, which is almost as great as the entire population, males and females, in the United States.

Starting in 1999, law enforcement agencies in both Great Britain and the United States began switching to a new version of RFLP analysis using shorter sequences called **STRs** ("**Short Tandem Repeats**"). STRs are repeated sequences of a few (usually four) nucleotides, e.g., TCATTCATTCATTCAT. They often occur in the untranslated parts of known genes (whose sequence can be used for the PCR primers). The exact number of repeats (6, 7, 8, 9, etc.) varies in different people (and, often, in the gene on each chromosome; that is, people are often heterozygous for the marker).

When 13 STR loci — scattered over different chromosomes — are examined, the chance that two people picked at random have the same pattern is less than 1 in 1 **trillion**. The U.S. Federal Bureau of Investigation (FBI) wants to increase the number of loci examined to 20 further eliminating the possibility of false positives.

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11.8: Gel Blotting

Gel blotting is a technique for visualizing a particular subset of macromolecules — proteins, or fragments of DNA or RNA — initially present in a complex mixture. The steps:

1. Separate the molecules by electrophoresis. This is done in a gel which allows the molecules to migrate under the influence of the electric field.
2. "Blot" them with a nitrocellulose filter. For unknown reasons, the molecules stick tightly to the filter and will retain their relative positions when flooded with fluid at the next step.
3. Bathe the filter with a solution containing a "probe": a molecule that will combine specifically with the target molecules; that is, the one(s) you are looking for and carries a mean of visualization, e.g. a radioactive or fluorescent marker.

Southern Blot

The diagram illustrates the procedure for detecting **DNA** fragments containing a particular sequence. DNA is extracted from the cell and is partially digested by a restriction endonuclease. The resulting DNA fragments are separated by electrophoresis and then denatured to form single-stranded molecules (ssDNA). Without altering their positions, the separated bands of ssDNA are transferred to a nitrocellulose filter and exposed to radiolabeled cDNA or RNA. If the probe detects complementary DNA sequences, it will bind to them. The presence of the probe in a particular band is revealed by autoradiography.

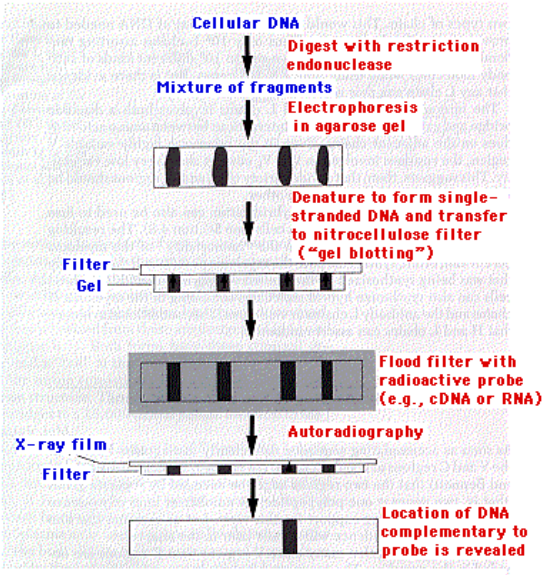


Figure 11.8.1: Southern Blotting

This procedure was developed by E. M. Southern and the finished product is called a "Southern blot". The same basic procedure can also be used to separate and visualize RNA molecules and protein molecules. As a humorous extension of the term "Southern blot", these have been dubbed "Northern" and "Western" blots, respectively (Table 11.8.1).

Table 11.8.1: Summary or Common Gel Blots

Type of Blot	Molecules separated by electrophoresis	Probe
Southern	ssDNA	cDNA or RNA
Northern	denatured RNA	RNA or cDNA
Western	Protein	Antibodies

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11.9: Genetic Screening for Phenylketonuria

Phenylketonuria is one of the commonest inherited disorders — occurring in approximately 1 in 10,000 babies born in the U. S. It occurs in babies who inherit two mutant genes for the enzyme **phenylalanine hydroxylase** (**PAH** — "1" in the figure on the left). This enzyme normally starts the process of breaking down molecules of the amino acid phenylalanine that are in excess of the body's needs for protein synthesis.

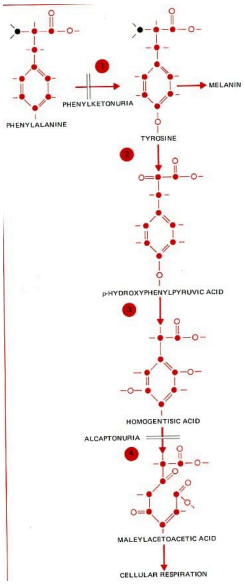


Figure 11.8.1 Phenylalanine pathways

The complete pathway is shown in the above figure. Phenylalanine that is in excess of the body's needs for protein synthesis is broken down as shown here and used in cellular respiration and to synthesize melanin as needed. Carbon atoms are shown in color, nitrogen atoms in black, and hydrogen atoms as short dashes.

Because we inherit two copies of the gene for the enzyme, both must be defective to produce the disease. A laboratory test that measures how quickly an injection of phenylalanine is removed from the blood can distinguish a person who has one PKU gene from a person who has none, but the person with one is perfectly healthy because the unmutated allele produces enough of the enzyme. However, these heterozygous individuals are "carriers" of the disease.

The Phenylalanine Tolerance Test

A short time after administering a measured amount of phenylalanine to the subject, the concentration of phenylalanine in the blood plasma is measured. The level is usually substantially higher in people who carry one PKU gene (even though they show no signs of disease) than in individuals who are homozygous for the unmutated gene. Both parents must be heterozygous (i.e., must be "carriers" of the trait) to produce a child with PKU. The chance of their doing so is 1 in 4.

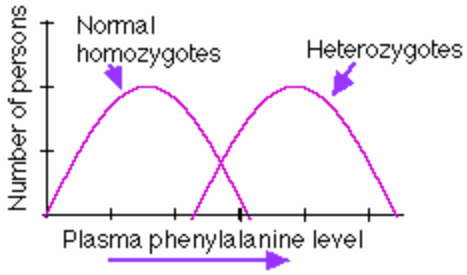


Figure 11.8.2 Phenylaline Tolerance test

Inability to remove excess phenylalanine from the blood during infancy and early childhood produces a variety of problems including mental retardation. Fortunately, a simple test (needing only a drop of blood) done shortly after birth can identify the genetic defect and, with close attention to the amount of phenylalanine in their diet, the children can develop normally.

Alcaptonuria is another inherited disease involving this pathway. It results from the inheritance of two mutant genes for the enzyme ("4") that converts homogentisic acid to maleylacetoacetic acid. When step 4 is blocked, homogentisic acid accumulates in the blood. The kidney excretes this excess in the urine, and oxidation of homogentisic acid by the air turns the urine black. Diseases like PKU and alcaptonuria are called "inborn errors of metabolism" because they are inherited and each is characterized by a distinct metabolic defect.

Genetic Screening

In the United States, approximately 1 person in 50 has inherited a PKU allele. This means that some 5 million people in the U.S. are "carriers". Should they be tested before they decide to become parents? By testing the DNA of prospective parents, their genotype can be determined and their odds of producing an afflicted child calculated. In the case of PKU, if **both** parents are heterozygous for the gene, there is a 1 in 4 chance that they will produce a child with the disease.

Problems:

- Scores of different mutations in the PAH gene can cause the disease. A probe for one will probably fail to identify a second. A mixture of probes, one for each of the more common mutations, can be used. But there remains the problem of "false negatives": people who are falsely told they do not carry a mutant gene.
- With an effective treatment for PKU available, should heterozygous parents forego having children?
- Do they want their health insurance company to know their status?

Genetic Screening Example

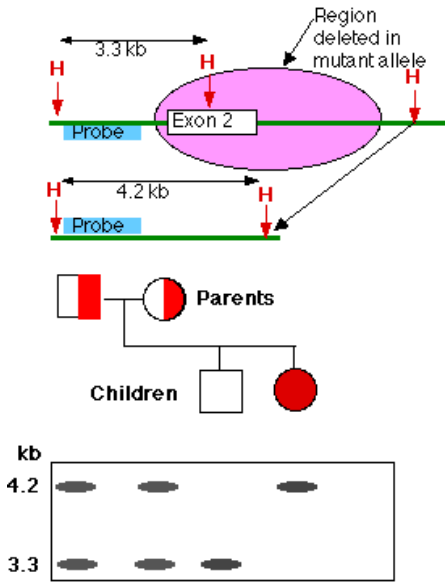


Figure 11.8.3 PKU. Genetic screening for "a" (NOTE: **not** "the" — many different mutations in the PKU gene have been identified) PKU allele.

- Top: schematic of a portion of the gene encoding the enzyme phenylalanine hydroxylase (PAH) showing the sites cut by the restriction enzyme HindIII ("H") and the region to which the radioactive probe binds a mutant version of the gene with a deletion that destroys its function. The deletion eliminates the HindIII site in Exon 2, lengthening the DNA fragment to which the probe binds from 3.3 to 4.2 thousand base pairs (kb) (and thus revealing a RFLP).
- Middle: The daughter (solid circle) with PKU inherited one PKU allele from each of her parents (half-filled symbols). Her brother (open square) beat the odds (0.5) of inheriting at least one PKU allele and thus of being a carrier. If he had been a carrier, would he have wanted to know?
- Bottom: The blot shows the fragments to which the probe binds directly beneath each individual in the pedigree. It reveals only the 3.3 kb fragment for the brother, only the 4.2 kb fragment for the sister, and both for each parent.

The particular mutation in this family is only one of many mutant PAH alleles that cause PKU, and testing with this particular enzyme and probe would not necessarily detect the others.

Phenylalanine hydroxylase (PAH) is made in the liver

The evidence:

- A child with PKU was cured when he received a transplanted liver (needed for reasons unrelated to his PKU). (Described by Vajro, P., et. al., in the **New England Journal of Medicine 329**:363, 29 July 1993.)
- Hepatic Nuclear Factor 1 (HNF1) is a **transcription factor** that is strongly expressed in the cells of the liver (called hepatocytes). In these cells, it binds to and activates the promoters of many genes expressed in the liver. "Knockout" mice were created from embryonic stem (ES) cells carrying two mutant PAH genes.Among other problems, these mice produced no PAH and had severe PKU. (Described by Pontoglio, M., et. al., in **Cell 84**:575, 23 February 1996.)

Dominant or recessive

The disease PKU is clearly inherited as a recessive trait. Only if one inherits a mutant allele from each parent will one develop the disease. However, heterozygous people are easily distinguished from homozygotes by the phenylalanine tolerance test. So using the test as the criterion, the PKU allele shows partial dominance. So, the relationship between genotype and phenotype is not always straightforward. What is the criterion of phenotype in this case? the disease? the results of the tolerance test?

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11.10: Antisense RNA

Messenger RNA (mRNA) is single-stranded. Its sequence of nucleotides is called "sense" because it results in a gene product (protein). Normally, its unpaired nucleotides are "read" by transfer RNA anticodons as the ribosome proceeds to **translate** the message.

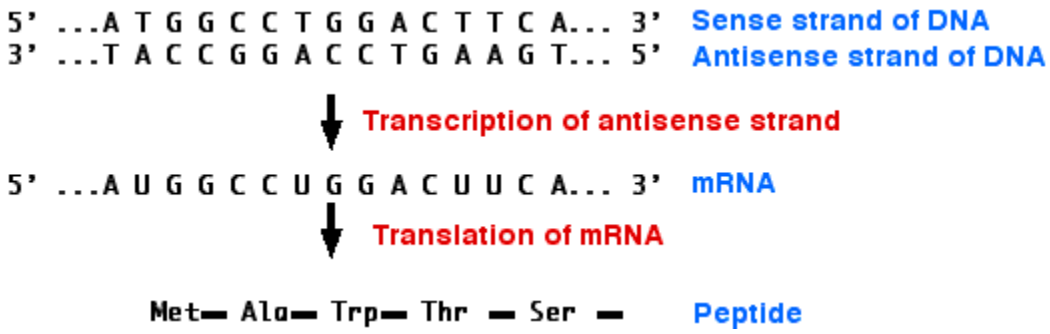


Figure 11.9.1 Sense Strand

However, RNA **can** form duplexes just as DNA does. All that is needed is a second strand of RNA whose sequence of bases is complementary to the first strand.

Example

5´ C A U G 3´ mRNA
3´ G U A C 5´ Antisense RNA

The second strand is called the **antisense strand** because its sequence of nucleotides is the complement of message sense.

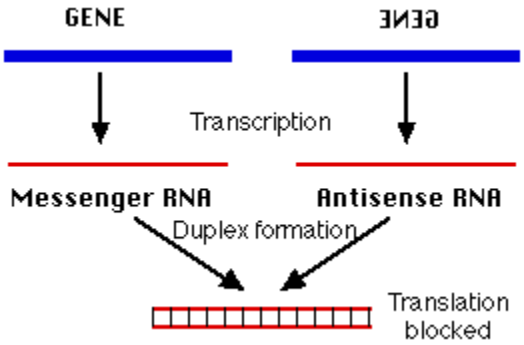


Figure 11.9.2 Antisense RNA

When mRNA forms a duplex with a complementary antisense RNA sequence, translation is blocked. This may occur because the ribosome cannot gain access to the nucleotides in the mRNA or because the duplex RNA is quickly degraded by ribonucleases in the cell. With recombinant DNA methods, synthetic genes (DNA) encoding antisense RNA molecules can be introduced into the organism.

Examples

The Flavr Savr tomato

Most tomatoes that have to be shipped to market are harvested before they are ripe. Otherwise, ethylene synthesized by the tomato causes them to ripen and spoil before they reach the customer. Transgenic tomatoes have been constructed that carry in their genome an artificial gene (DNA) that is transcribed into an antisense RNA complementary to the mRNA for an enzyme involved in ethylene production. These tomatoes make only 10% of the normal amount of the enzyme.

The goal of this work was to provide supermarket tomatoes with something closer to the appearance and taste of tomatoes harvested when ripe. However, these tomatoes often became damaged during shipment and handling and have been taken off the market.

Transgenic Tobacco



Fig.11.9.3 tobacco flower

Flower of a tobacco plant carrying a transgene whose transcript is antisense to one of the mRNAs needed for normal flower pigmentation.

Transgenic Flower



Figure 11.9.4 Transgenic flower

Flower of another transgenic plant that failed to have its normal pigmentation altered.

Making transgenic plants

There are several methods for introducing genes into plants, including

- infecting plant cells with plasmid vectors carrying the desired gene
- shooting microscopic pellets containing the gene directly into the cell

In contrast to animals, there is no real distinction between somatic cells and germline cells. Somatic tissues of plants, e.g., root cells grown in culture,

- can be transformed in the laboratory with the desired gene
- grown into mature plants with flowers.

If all goes well, the transgene will be incorporated into the pollen and eggs and passed on to the next generation.

In this respect, it is easier to produce transgenic plants than transgenic animals.

Antisense RNA also occurs naturally

Do cells contain genes that are naturally translated into antisense RNA molecules capable of blocking the translation of other genes in the cell? The answer is yes, and these seem to represent another method of **regulating gene expression**. In both mice and humans, the gene for the **insulin-like growth factor 2 receptor** (**Igf2r**) that is inherited from the father synthesizes an antisense RNA that appears to block synthesis of the mRNA for Igf2r. An inherited difference in the expression of a gene

depending on whether it is inherited from the mother or the father is called genomic or parental **imprinting**.

RNA interference (RNAi)

In testing the effects of antisense RNA, one should use **sense** RNA of the same coding region as a **control**. Surprisingly, preparations of sense RNA often turn out to be as effective an inhibitor as antisense RNA.

Why? It seems that the preparations of sense RNA often are contaminated with hybrids: sense and antisense strands that form a double helix of double-stranded RNA (**dsRNA**). Double-stranded RNA corresponding to a particular gene is a powerful suppressant of that gene. In fact, the suppressive effect of antisense RNA probably also depends on its ability to form dsRNA (using the corresponding mRNA as a template).

The ability of dsRNA to suppress the expression of a gene corresponding to its own sequence is called **RNA interference** (**RNAi**). It is also called **post-transcriptional gene silencing** or **PTGS**.

Mechanism of RNAi

The only RNA molecules normally found in the cytoplasm of a cell are molecules of single-stranded RNA. If the cell finds molecules of **double-stranded** RNA (**dsRNA**), it uses an enzyme called **Dicer** to cut them into fragments containing ~21 base pairs (~2 turns of a double helix). The two strands of each fragment then separate — releasing the **antisense strand**. With the aid of a protein, it binds to a **complementary sense sequence** on a molecule of **mRNA**. If the base-pairing is exact, the mRNA is destroyed. Because of their action, these fragments of RNA have been named "small (or short) interfering RNA" (**siRNA**). The complex of siRNA and protein is called the "**R**NA-**i**nduced **s**ilencing **c**omplex" (**RISC**).

siRNAs can also interfere with transcription

There is growing evidence that siRNAs can also inhibit the transcription of genes

- perhaps by binding to complementary sequences on **DNA** or
- perhaps by binding to the nascent RNA transcript as it is being formed.

In fission yeast, at least, the siRNA is complexed with one molecule of each of three different proteins. The entire complex is called the **RITS** complex ("**R**NA-**i**nduced initiation of **t**ranscriptional gene **s**ilencing")

How these siRNAs — synthesized in the cytosol — gain access to the DNA in the nucleus is unknown.

Synthetic siRNA molecules that bind to gene promoters can — in the laboratory — repress transcription of that gene. The repression is mediated by methylation of the DNA in the promoter and, perhaps, methylation of histones in the vicinity.

There is a strain of rice (LGC-1) that produces abnormally low levels of proteins called **glutelins**. It turns out that of several glutelin genes found in rice

- two closely-similar glutelin genes are located back to back on the same chromosome.
- In LGC-1, a deletion has occurred between the two genes which removes the signal that would normally stop transcription after the first gene.
- Thus RNA polymerase II transcribes right past the first gene and on into the second.
- The result is a messenger RNA with almost-identical sequences running in opposite directions.
- This causes the mRNA to fold up into a molecule of **double-stranded RNA** (dsRNA).
- A Dicer-like enzyme cuts up the dsRNA into small interfering RNAs (siRNAs) that suppress further transcription of those genes as well as other glutelin genes.

Why RNAi?

RNAi has been found to operate in such diverse organisms as plants, fungi, and animals such as ***Drosophila melanogaster***, ***Caenorhabditis elegans***, and even mice and the zebrafish. Such a universal cell response must have an important function. What could it be?

Some possibilities:

- Some viruses of both plants and animals have a genome of dsRNA. And many other viruses of both plants and animals have an RNA genome that in the host cell is briefly converted into dsRNA. So RNAi may be a weapon to counter infections by these viruses by destroying their mRNAs and thus blocking the synthesis of essential viral proteins.
- Transposons may be transcribed into RNA molecules with regions that are double-stranded. RNAi could then destroy these.
- RNA interference may be the unexpected dividend of another basic process of controlling gene expression.

RNAi as a tool

In any case, the discovery of RNAi adds a promising tool to the toolbox of molecular biologists. Introducing dsRNA corresponding to a particular gene will knock out the cell's own expression of that gene. (Feeding ***C. elegans*** on ***E. coli*** manufacturing the dsRNA will even do the trick.)

Heroic Example

In the 24 March 2005 issue of **Nature**, Sönnichsen *et al* reported that they have injected dsRNAs corresponding to 20,326 of C. elegans's genes (98% of the total!) and monitored the effect of each on embryonic development from the completion of meiosis (following fertilization) through the second mitotic division that produces the 4-cell embryo.

They found that at least 661 different genes altered some process during this period:

- about half of them involved in cell division and
- half in general cell metabolism.

(Another thousand genes produced phenotypic effects that were seen at later stages of development.)

Because RNAi can be done in particular tissues at a chosen time, it often provides an advantage over conventional gene "knockouts" where the missing gene is carried in the germline and thus whose absence may kill the embryo before it can be studied.

Another Example: screening genes for their effect on drug sensitivity

- Distribute your cells in thousands of wells and add — from a "library" of thousands of siRNAs representing the entire genome — siRNA molecules targeting the expression of one gene to each well
- Add the drug to all the wells
- See which wells have cells that respond

Some other promising applications of RNAi

In mammalian cells

In mammalian cells, introducing dsRNA fragments only reduces gene expression temporarily. However, mammalian cells can be infected with a **DNA vector** that encodes an RNA molecule of 50–80 nucleotides called a "small hairpin RNA" (**shRNA**) containing a sequence corresponding to the gene that one wishes to suppress. As the shRNA is synthesized, dicer converts it into a typical siRNA molecule. Because the cell can continuously synthesize shRNA, the interference is long-lasting. In fact, with vectors that become integrated in the host genome, RNAi can be passed on to the descendants.

In plants

The 19 June 2003 issue of **Nature** reported on coffee plants that were engineered to express a transgene that makes siRNA that interferes — by RNAi — with the expression of a gene needed to make **caffeine**. So perhaps "decaf" coffee will one day no longer require the chemical removal of caffeine from coffee beans.

Monsanto is developing a transgenic corn (maize) that expresses a dsRNA corresponding to the sequence of an essential gene in the western corn rootworm, a devastating pest of the crop. After ingesting this dsRNA, the insect's own cells process it into an siRNA that targets the gene's mRNA for destruction and kills the worm in a few days.

Amplification of RNAi

In *C. elegans*, plants, and *Neurospora*, the introduction of a few molecules of dsRNA has a potent and long-lasting effect. In plants, the gene silencing spreads to adjacent cells (through plasmodesmata) and even to other parts of the plant (through the phloem). RNAi within a cell can continue after mitosis in the progeny of that cell. Triggering of RNAi in *C. elegans* can even pass through the germline into its descendants.

Such amplification of an initial trigger signal suggests a catalytic effect. It turns out that these organisms have RNA-dependent RNA polymerases (RdRPs) that uses the mRNA targeted by the initial antisense siRNA as a template for the synthesis of more siRNAs. Synthesis of these "secondary" siRNAs even occurs in adjacent regions of the mRNA. So not only can these secondary siRNAs target additional areas of the original mRNA, but they are potentially able to silence mRNAs of other genes that may carry the same sequence of nucleotides.

This phenomenon, called "transitive RNAi",

- may complicate the interpretation of gene suppression experiments as the expression of other genes may be suppressed in addition to the target gene;
- raises a warning flag for the use of RNAi to suppress single genes in human therapy (although RdRPs and amplification have not been observed in mammalian cells).

RNAi in human therapy

Because its target is so specific, the possibility of using RNAi to shut down the expression of a single gene has created great excitement that a new class of therapeutic agents is on the horizon. Many clinical trials are underway exploring the use of siRNA molecules in the treatment of a wide variety of diseases. To date, the most promising results have been using RNAi to target an inherited disease in which the liver secretes a mutant form of transthyretin leading to the accumulation of amyloid deposits in neurons and elsewhere.

MicroRNAs (miRNAs)

In ***C. elegans***, successful development through its larval stages and on to the adult requires the presence of at least two "**microRNAs**" ("miRNAs") — single-stranded RNA molecules containing about 22 nucleotides and thus about the same size as siRNAs.

These small **single-stranded** transcripts are generated by the cleavage of larger precursors using the *C. elegans* version of Dicer.

They act by either destroying or **inhibiting translation** of several messenger RNAs in the worm (usually by binding to a region of complementary sequence in the 3' untranslated region [3'-UTR] of the mRNA).

The microRNAs (miRNAs) in C. elegans (which were first called "small temporal RNAs") turn out to be representatives of a large class of RNAs that are encoded by the organism's **own genes**.

- The initial product of gene transcription is a large molecule called **pri-miRNA**.
- While still within the nucleus an enzyme called **Drosher** cuts the pri-miRNA into a shorter molecule (~70 nucleotides) called **pre-miRNA**.
- The pre-miRNA is exported into the cytosol where it is cleaved (by **Dicer** in animals) into the miRNA.

MicroRNAs

- are found in all animals (humans generate some 1000 miRNAs) and plants but not in fungi.
- contain 19–25 nucleotides;
- are encoded in the genome
  - some by stand-alone genes (that may encode several miRNAs)
  - some by portions of an intron of the gene whose mRNA they will regulate.
- may be expressed in
  - only certain cell types and
  - at only certain times in the differentiation of a particular cell type.

While direct evidence of the function of many of these newly-discovered gene products remains to be discovered, they regulate gene expression by regulating messenger RNA (mRNA), either

- destroying the mRNA when the sequences match exactly (the usual situation in plants) or
- repressing its **translation** when the sequences are only a partial match. In this latter case, it probably requires several miRNAs to bind simultaneously in the 3'-UTR.

MicroRNAs have two traits ideally suited for this:

- Being so small, they can be rapidly transcribed from their genes.
- They do not need to be translated into a protein product to act (in contrast, e.g., to transcription factors).

MicroRNAs regulate (repress) expression of genes in mammals as well. Genome analysis has revealed thousands of human genes whose transcripts (**m**RNAs) contain sequences to which one or more of our **mi**RNAs might bind. Probably each miRNA can bind to as many as 200 different mRNA targets while each mRNA has binding sites for multiple miRNAs. Such a system provides many opportunities for coordinated mRNA translation.

A study reported in **Nature** (Lim, *et al.*, **433**: 769, 17 Feb 2005) used DNA chip analysis to show that when a particular miRNA was expressed in HeLa cells,

- a miRNA normally expressed in the brain repressed mRNA production by 174 different genes while
- a miRNA normally expressed in cardiac and skeletal muscle repressed mRNA production by 96 genes — all but 8 of them different from the those repressed by the brain miRNA.

As work proceeds rapidly in this field, the pattern that begins to emerge is that:

- Many genes — especially those involved in such housekeeping activities (e.g., cellular respiration) common to all cells — do not have 3'-UTRs that can be blocked by any of the miRNAs encoded in the genome.
- The genes that must be expressed in a particular type of differentiated cell and/or at a particular time in the life of that cell
  - do not express any of the miRNA genes that could block their expression but
  - do express miRNA genes that block the expression of other genes for specialized functions that would not be appropriate in that cell at that time.
- Rather than being simple switches that turn gene expression on or off, miRNAs seem to exert a more subtle effect — raising or lowering the level of gene expression (much as protein transcription factors do).

Thus repression of gene expression by miRNAs appears to be a mechanism to ensure regulated and coordinated gene expression as cells differentiate along particular paths. For example, when zygote genes begin to be turned on in the zebrafish blastula, one of them encodes a miRNA that triggers the destruction of the maternal mRNAs that have been running things up to then.

So miRNAs may play as important role as transcription factors in regulating and coordinating the expression of multiple genes in a particular type of cell at particular times.

Therapeutic miRNAs?

The ease with which miRNAs can be introduced into cells and their widespread effects on gene expression have given rise to hopes that they might be useful in controlling genetic disorders, e.g., cancer.

To date, some laboratory studies have been quite promising.

- A miRNA that blocks the expression of G1 and S-phase cyclins — and thus stops the cell cycle in its tracks — protects mice from liver cancer.
- a miRNA that inhibits genes needed for metastasis suppresses the metastasis of treated human breast cancer cells.

Summary

In addition to protein transcription factors, eukaryotes use **small RNA** molecules to regulate gene expression — almost always by repressing it — so the phenomenon is called **RNA silencing**.

There are two sources of small RNA molecules:

- small interfering RNAs (**siRNA**s)
  - Plant cells make these from the double-stranded RNA (dsRNA) of invading viruses.
  - Scientists and pharmaceutical companies make these as agents to turn off the expression of specific genes (called RNA interference or RNAi).
- micro RNAs (**miRNA**s)
  - These are encoded in the genomes of all plants and animals.
- Both siRNAs and miRNAs are processed in the same way in the cytosol of the cell.
  - Both are generated by Dicer.
  - Both are incorporated into an **R**NA-**i**nduced **s**ilencing **c**omplex (**RISC**).
    - If the nucleotide sequence of the small RNA exactly matches that of the mRNA, the mRNA is cut and destroyed.
    - If there is only a partial match (usually in its 3' UTR), translation (i.e., protein synthesis) is repressed. Both of these activities take place in the cytosol — perhaps in **P bodies**.
    - However, for some small RNAs, the RISC complex enters the nucleus and turns off transcription of the corresponding gene(s) by
      - binding to the unwound DNA sequence (or perhaps the RNA transcript as it is being formed)
      - converting euchromatin to heterochromation
      - methylating of lysine-9 histone H3 in the nucleosomes around the gene(s)

Aside from their use as laboratory — and perhaps therapeutic — tools, small RNAs are clearly essential to the organisms that make them.

Some examples:

- Plants and animals use them to defend themselves against viruses.
  - Example: When human cells are infected by hepatitis C virus (HCV), they produce miRNAs that interfere with gene expression by this RNA virus and thus its ability to replicate.
- Some herpesviruses use them to keep their host cell alive long enough to complete viral replication (by blunting a host immune response against the infected cell and preventing its premature death by apoptosis).
- Of the 46 miRNAs expressed in the Drosophila embryo, 25 have been shown to be essential to normal development.
- Correct embryonic development in other animals (e.g., *C. elegans*, zebrafish, mice) also requires them.
- They protect against the danger of mutations caused by transposons moving around in the genome.
- They are also needed to regulate the size of the pool of at least some types of stem cells.
- Transgenic mice with a single miRNA gene knocked out develop severe immunodeficiency affecting dendritic cells, helper T cells, and B cells.
- Reduced, or no, expression of certain miRNAs are characteristic of several different cancers in humans.

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11.11: Antisense Oligodeoxynucleotides and their Therapeutic Potential

Antisense oligonucleotides are synthetic polymers. The monomers are chemically-modified **deoxynucleotides** like those in DNA or ribonucleotides like those in RNA. There are usually only 15–20 of them, hence "oligo". Their sequence (3′ → 5′) is antisense; that is, complementary to the sense sequence of a molecule of **mRNA**.

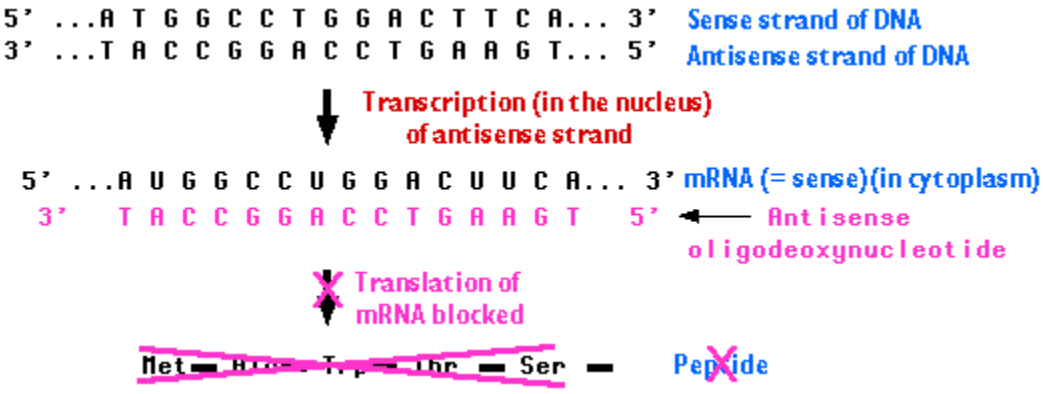


Figure 11.10.1 Antisense oligonucleotides

Antisense oligonucleotides are synthesized in the hope that they can be used as therapeutic agents — blocking disease processes by altering the synthesis of a particular protein. This would be achieved by the binding of the antisense oligonucleotide to the mRNA from which that protein is normally synthesized. Binding of the two may

- physically block the ability of ribosomes to move along the messenger RNA preventing synthesis of the protein;
- hasten the rate at which the mRNA is degraded within the cytosol;
- prevent splicing errors that would otherwise produce a defective protein.

To be useful in human therapy, antisense oligonucleotides must be able to enter the target cells; avoid digestion by nucleases; and not cause dangerous side-effects. To achieve these goals, antisense oligonucleotides are generally chemically modified to resist digestion by nucleases and attached to a targeting device such as the ligand for the type of receptors found on desired target cells or antibodies directed against molecules on the surface of the desired target cells.

Antisense Oligonucleotides Uses

A variety of antisense oligonucleotides are being tested as possible weapons against:

- Hepatitis C virus (HCV). Successful infection of the liver by this virus requires that the liver produce a particular microRNA (miRNA-122). Injections of HCV-infected humans with an ODN ("miravirsen") complementary to miRNA-122 suppresses the virus.
- **HIV-1**, the most frequent cause of **AIDS** in the United States
- Ebola virus, the cause of the often-fatal Ebola hemorrhagic fever
- **human cytomegalovirus** (**HCMV**); which frequently causes serious complications in AIDS patients
- **asthma**; inhalation of an antisense oligonucleotide targeting the mRNA of GATA3 (a transcription factor that promotes Th2 responses) provides relief to patients with allergic asthma.
- certain **cancers**, e.g., chronic myelogenous leukemia (CML)
- certain types of **inflammation** caused by cell-mediated immune reactions
- Duchenne muscular dystrophy (DMD)
- familial hypercholesterolemia — targets the mRNA for apolipoprotein B-100. On 31 January 2013, the antisense ODN mipomersen (Kynamro®) received regulatory approval for use in humans with familial hypercholesterolemia.

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11.12: Forward and Reverse genetics

The Zebrafish

The zebrafish, *Danio rerio*, has become another popular "model" organism with which to study fundamental biological questions. It is a small (1–1.5 inches)(2.5–3.8 cm) freshwater fish that grows easily in aquaria (it is available at many pet stores). Some of its advantages for biologists:

- It breeds early and often (daily).
- It is a vertebrate, like us, and thus can provide clues to human biology that invertebrates like *Drosophila* and *Caenorhabditis elegans* may not.
- Its embryos, like those of most fishes, develop outside the body where they can be easily observed (unlike mice).
- Its embryos are transparent so defects in development can be seen easily.
- Individual cells in the embryo can be labeled with a fluorescent dye and their fate followed.
- Embryonic development is quick (they hatch in two days).
- They can absorb small molecules, such as mutagens, from the aquarium water.
- Individual cells — or clusters of cells — can be transplanted to other locations in the embryo (as Mangold did with newt embryos).
- They can be forced to develop by parthenogenesis to produce at will homozygous animals with either:
  - a male-derived or
  - female-derived genome.
- They can be cloned from somatic cells.
- They can be made transgenic (like mice and *Drosophila*)
- Its genome (1.4×10^9 base pairs) has been sequenced revealing 26,606 protein-coding genes.

Forward Genetics

Since Mendel's time, most genetics has involved observing an interesting phenotype and tracking down the gene responsible for it. So this "forward" genetics proceeds from phenotype -> genotype. Some examples in these pages:

- Mendel's work
- RFLP analysis of large families
- the one gene - one enzyme theory

These methods have been called "forward" genetics to distinguish them from a more recent approach, which has become an urgent priority with the successes of genome sequencing.

Reverse Genetics

Rapid methods of DNA sequencing has generated a vast amount of data. Thousands of suspected genes have been revealed (e.g., finding *open reading frames* — ORFs), but the function of many of them is still unknown. But now with a knowledge of the DNA sequence of a gene of unknown function, one can use methods for suppressing that particular gene ("knockdown"), and then observe the effect on the phenotype.

So this "reverse" genetics proceeds from genotype -> phenotype. Reverse genetics has been applied successfully to plants; mice; *C. elegans*; and can also be used with the zebrafish. For example, the function of a mysterious gene sequence in *Danio* can be studied by

- synthesizing a short antisense oligonucleotide complementary to a section of the gene.
- The oligonucleotide is chemically-modified to make it more stable than a fragment of RNA.
- Binding to its complementary sequence on the messenger RNA (mRNA) produced by transcription of the animal's gene, blocks ("knocks down") gene expression by preventing translation or disrupting normal splicing of the mRNA.

Because we share so many similar gene sequences (orthologous genes) with *Danio*, if one can discover the function of the gene in *Danio*, then we have a better idea of the role of its ortholog in humans.

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11.13: Metagenomics

All the genomes listed on my page Genome Sizes describe the complete genome of a single species. For bacteria and archaeons, this means that the organism was grown in pure culture to provide the DNA for sequencing. But it is now clear that the microbial world contains vast numbers of both groups that have never been grown in the laboratory and thus have escaped study. Soil, water, and the contents of our large intestine are examples of habitats that teem with unknown microorganisms.

Thanks to the recent development of sequencing machines capable of rapidly (and inexpensively) sequencing huge amounts of DNA, it is now practical to sequence the DNA extracted from complex microbial ecosystems like that found in a soil sample. Several different approaches are used, but all depend on a first step of extracting the microbial DNA from the sample (and separating it from the far more complex DNA of any eukaryotes that may be present).

Assessing Microbial Diversity

The DNA encoding the small subunit (16S) of the ribosomes of both bacteria and archaeons contain some highly conserved regions; that is, regions of identical or almost identical sequence. Using primers that target these regions, one can then produce enough material by the polymerase chain reaction PCR to sequence the entire 16S rRNA gene.

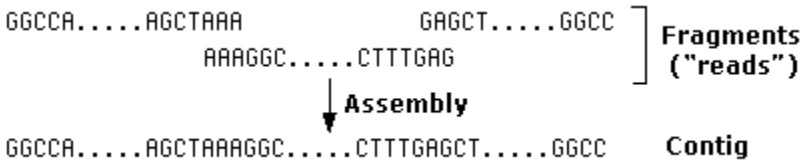
Comparing the various sequences to a database of sequences from known organisms, one can estimate how many different types of microbes are present. Because of the substantial genetic diversity found between "strains" of a single species (e.g., *E. coli* K-12 and *E.coli* O157:H7), closely-related (> 97% identity) 16S rDNA sequences are assigned to a single "**phylotype**" because we cannot be sure whether they belong to separate species or to two strains of the same species. In either case, the collection of 16S rDNA sequences can be arranged to form a phylogenetic tree to show the patterns of relatedness.

Cataloging the Genes in a Microbial Ecosystem

Analyzing the 16S rDNA genes in a sample tells us who is there, but, of course, is not a complete genome and tells us nothing about the other genes present in the various members of the population. This information can be gained by "shotgun" sequencing of the environmental DNA sample.

The Steps:

- Break the DNA in short fragments.
- Insert these into a vector, e.g. a plasmid capable of growing in *E. coli* K-12.
- Expose *E. coli* cells to this random mix and grow the individual bacterial cells into colonies.
- The result: a **library** containing millions of random DNA fragments from the original sample.
- Isolate the plasmids and sequence them. Sequence "reads" average around 100 nucleotides — far shorter than a gene but often enough to move on to the next step.
- Use a powerful computer to attempt to assemble the fragments into a linear sequence of DNA. The computer looks for identical stretches of nucleotides in different fragments and uses the overlap to assemble them into a "**contig**".



- Look (have the computer look) for open reading frames (ORFs) of protein-encoding genes.
- Compare the ORFs with those of known microbes already in databases to see if a function can be deduced.

The sheer diversity of organisms in most microbial ecosystems makes it virtually impossible to find enough contigs to assemble a complete genome for any one organism like those listed in Genome Sizes. What you get instead is a window into the many kinds of genes present in one inhabitant or another of that ecosystem. For example, you may discover genes that encode proteins able

to degrade environmental pollutants or genes able to synthesize a new antibiotic.

Finding New Functions in Microbial Populations

Another way of exploiting metagenomics is to look for new functions in the host (e.g. *E. coli*) if it can express the new gene with which it was transformed. For example, screening the library of *E. coli* clones for the ability to resist an antibiotic can reveal genes involved in antibiotic resistance — a worrisome development in recent years.

Some Applications of Metagenomics

The Sargasso Sea

Metagenomic analysis of the DNA extracted from sea water in the Sargasso Sea revealed the presence of over a thousand different 16S rDNA genes (and thus approximately that number of different species) and over a million protein-encoding genes.

The Human Colon

0.3 g fecal samples from two healthy humans produced 78 million base pairs of sequence. Each subject produced some 25 thousand open reading frames (ORFs) of which about half could be recognized as already-known bacterial or archaeal genes. Included were genes encoding enzymes for the synthesis of vitamins (e.g., vitamin B_1), amino acids, and enzymes for the digestion of complex polysaccharides in our diet which would otherwise be indigestible. Perhaps as much as 10% of the energy we extract from our food is made available to us by the activity of these microorganisms.

Acid Mine Drainage

Metagenomic analysis of the acidic water (pH ~0.5) flowing from an abandoned metal mine in California revealed a much simpler ecosystem than those described above: only 3 species of bacteria and 2 of archaea. With such limited diversity, it was possible to assemble almost-complete genomes for two of these organisms.

A South African Gold Mine

Simpler still was the ecosystem found in water 2.8 km (1.7 miles) down in a gold mine. Only one organism turned up: an autotrophic bacterium capable of extracting energy from inorganic substances in its environment and synthesizing all the molecules needed for its life from them.

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UNIT 12: CANCER

A cancer is an uncontrolled proliferation of cells. In some the rate is fast; in others, slow; but in all cancers the cells never stop dividing. This distinguishes cancers — malignant tumors — from benign growths like moles where their cells eventually stop dividing (usually). Even more important, benign growths differ from malignant ones in not producing metastases; that is, they do not seed new growths elsewhere in the body.

12.1: Cancer in General

12.2: Cancer Cells in Culture

12.3: Oncogenes

12.4: Tumor Suppressor Genes

12.5: BCL-2

12.6: Burkitt's Lymphoma

12.7: Chronic Myelogenous Leukemia (CML)

12.8: Fighting Cancer with Inhibitors of Angiogenesis

12.9: Immunotherapy of Cancer

12.10: Cancer- The Causes and Prevention of Cancer

12.11: Estimating Cancer Risks

12.12: The LD50 test

12.13: Dioxin

12.14: Magnetic Fields and Cancer

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12.1: Cancer in General

A cancer is an uncontrolled proliferation of cells. In some the rate is fast; in others, slow; but in all cancers the cells never stop dividing. This distinguishes cancers — malignant tumors — from benign growths like moles where their cells eventually stop dividing (usually). Even more important, benign growths differ from malignant ones in not producing metastases; that is, they do not seed new growths elsewhere in the body.

Cancers are **clones**. No matter how many trillions of cells are present in the cancer, they are all descended from a single ancestral cell. Evidence: Although normal tissues of a woman are a mosaic of cells in which one X chromosome or the other has been inactivated, all her tumor cells — even if from multiple sites — have the same X chromosome inactivated.

Cancers begin as a primary tumor. Most (maybe all) solid tumors shed cells into the lymph and blood. Most of these lack the potential to develop into tumors. However, some of the shed cells are able to take up residence and establish secondary tumors — **metastases** — in other locations of the body. These metastases, not the primary tumor, are what usually kills the patient.

Cancer cells are usually less differentiated than the normal cells of the tissue where they arose. Many people feel that this reflects a process of **dedifferentiation**, but I doubt it. Rather, evidence is accumulating that cancers arise in precursor cells — stem cells or "progenitor cells" — of the tissue: cells that are dividing by mitosis producing daughter cells that are not yet fully differentiated.

> A cancer is an uncontrolled proliferation of cells.

Cancer is a Genetic Disease

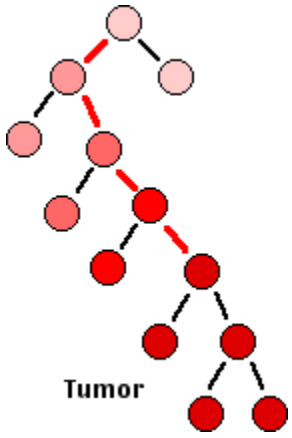


Figure 12.1.1 Cancer Gene Mutation

What probably happens is:

- A single cell — perhaps an adult stem cell or progenitor cell — in a tissue suffers a mutation (red line) in a gene involved in the cell cycle, e.g., an oncogene or tumor suppressor gene.
- This results in giving that cell a slight growth advantage over other dividing cells in the tissue.
- As that cell develops into a clone, some if its descendants suffer another mutation (red line) in another cell-cycle gene.
- This further deregulates the cell cycle of that cell and its descendants.
- As the rate of mitosis in that clone increases, the chances of further DNA damage increases.
- Eventually, so many mutations have occurred that the growth of that clone becomes completely unregulated.
- The result: full-blown cancer. (Genetic analysis reveals an average of 63 mutations in pancreatic cancers; almost as many in one type of adult brain cancer, but only 11 somatic mutations in a case of brain cancer in a child.)
- Sequencing samples from several areas in a primary tumor, as well as from some of its metastases, reveals a different collection of mutations from sample to sample. This finding is reinforced by the sequencing of the genome of **individual cells** from a single tumor each of which shows a unique pattern of shared and unique mutations. (The ability to sequence the genome of a single cell reveals that even normal cells in an adult have accumulated a suite of somatic mutations that differs from cell to cell. However, the rate of somatic mutations in these normal cells is only a fourth of that in cancer cells.)

So even though all the malignant cells in a cancer are descended from a single original cell — and thus are members of a single clone — they are no longer genetically-identical. As the tumor develops, its various cells develop a variety of additional mutations, and these give rise to "**subclones**" of varying degrees of malignancy with varying

- propensity to metastasize;
- susceptibility to treatment by anticancer drugs;
- propensity to relapse after apparently-successful therapy.

These findings should stimulate a reexamination of the use of chemotherapy.

- While chemotherapy may wipe out dominant subclones in a tumor, there is evidence that is also exerts a selective pressure for the expansion of more malignant, previously-minor, subclones.
- Most chemotherapeutic agents damage DNA so while killing off some cells, they will raise the mutation rate in any surviving cells perhaps encouraging the outgrowth of even more malignant subclones.

Evidence: In a group of patients with chronic lymphocytic leukemia, those receiving chemotherapy survived for shorter periods than those that did not.

Cancer Stem Cells

Stem cells are cells that divide by mitosis to form either two stem cells, thus increasing the size of the stem cell "pool", or one daughter that goes on to differentiate, and one daughter that retains its stem-cell properties. There is growing evidence that most of the cells in leukemias, breast, brain, skin, ovarian, and colon cancers are **not** able to proliferate out-of-control (and to metastasize). Only those members of the clone that retain their stem-cell-like properties (~2.5% of the cells in a tumor of the colon) can do so.

There is a certain logic to this. Most terminally-differentiated cells have limited potential to divide by mitosis and, seldom passing through S phase of the cell cycle, are limited in their ability to accumulate the new mutations that predispose to becoming cancerous. Furthermore, they often have short life spans — being eliminated by apoptosis (e.g., lymphocytes) or being shed from the tissue (e.g., epithelial cells of the colon). The adult stem cell pool, in contrast, is long-lived, and its members have many opportunities to acquire new mutations as they produce differentiating daughters as well as daughters that maintain the stem cell pool.

Colon cancer

- Begins with the development of **polyps** in the epithelium of the colon. Polyps are **benign** growths.
- As time passes, the polyps may get bigger.
- At some point, nests of malignant cells may appear within the polyps
- If the polyp is not removed, some of these malignant cells will escape from the primary tumor and metastasize throughout the body.

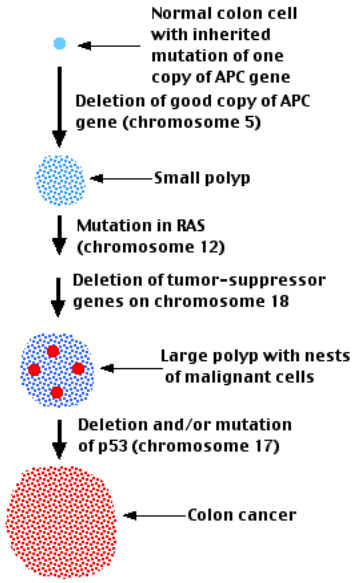


Figure 12.1.2 Colon Cancer

Examination of the cells at the earliest, polyp, stage, reveals that they contain one or two mutations associated with cancer. Frequently these include

- the deletion of a healthy copy of the ***APC*** (**a**denomatous **p**olyposis **c**oli) gene on chromosome 5 leaving behind a mutant copy of this tumor suppressor gene

  Two results:

  1. One of the functions of the APC gene product is to destroy the transcription factor β-catenin thus preventing it from turning on genes that cause the cell to divide. With no, or a defective, APC protein, the normal brakes on cell division are lifted.
  2. Another function of the APC protein is to help attach the microtubules of the mitotic spindle to the kinetochores of the chromosomes. With no, or a defective, APC product available, chromosomes are lost from the spindle producing aneuploid progeny.
- a mutant oncogene (often ***RAS***).
- deletion and/or mutation of the tumor suppressor gene ***p53***

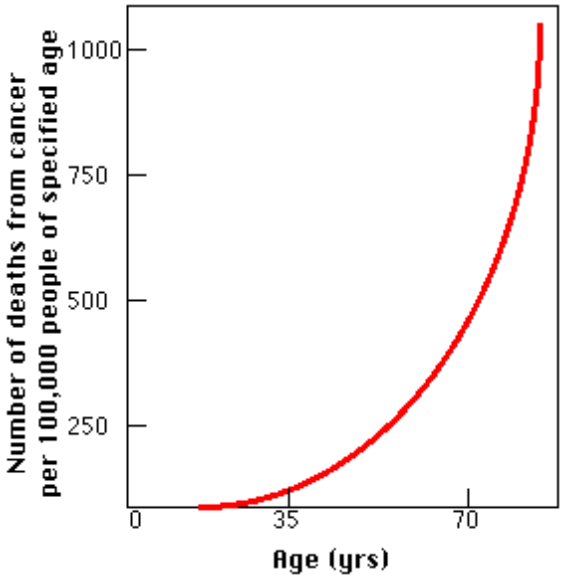


Figure 12.1.3 Death Rate due to cancer in the USA

The graph also explains why cancer has become such a common cause of death during the twentieth century. It probably has very little to do with exposure to the chemicals of modern living and everything to do with the increased longevity that has been such a remarkable feature of the 20th century. A population whose members increasingly survive accidents and infectious disease is a population increasingly condemned to death from such "organic" diseases as cancer.

Causes of Cancer

Cancers are caused by

- anything that **damages DNA**; that is anything that is **mutagenic**
  - radiation that can penetrate to the nucleus and interact with DNA
  - chemicals that can penetrate to the nucleus and damage DNA. Chemicals that cause cancer are called **carcinogens**.
- anything that stimulates the rate of **mitosis**. This is because a cell is most susceptible to mutations when it is replicating its DNA during the S phase of the cell cycle.
  - certain hormones (e.g., hormones that stimulate mitosis in tissues like the breast and the prostate gland)
  - chronic tissue injury (which increases mitosis in the stem cells needed to repair the damage)
  - agents that cause **inflammation** (which generates DNA-damaging **oxidizing agents** in the cell)
  - certain other chemicals; some the products of technology
  - certain **viruses**

  (Considering that from conception to death, an estimated 10^{16} mitotic cell divisions occur in humans, it is remarkable that cancer is not more common than it is.)

Viruses and Cancer

Many viruses have been studied that reliably cause cancer when laboratory animals are infected with them. What about humans? The evidence obviously is indirect but some likely culprits are:

- two **papilloma** viruses that can cause cancer of the cervix and other regions of the genitals (male as well as female).
- the **hepatitis B** and **hepatitis C** viruses, which infect the liver and are closely associated with liver cancer (probably because of the chronic inflammation they produce)
- some **herpes** viruses such as the **Epstein-Barr virus** (implicated in Burkitt's lymphoma) and KSHV that is associated with **Kaposi's sarcoma** (a malignancy frequently seen in the late stages of **AIDS**)
- two **h**uman **T**-cell **l**ymphotropic **v**iruses, **HTLV-1** and **HTLV-2**

But note that the viral infection only contributes to the development of cancer.

- Many people are infected by these viruses and do **not** develop cancer.
- When cancers do arise in infected people, they still follow our rule of clonality. Many cells have been infected, but only one (usually) develops into a tumor.

So again it appears that only if an infected cell is unlucky enough to suffer several other types of damage will it develop into a tumor.Nevertheless, widespread vaccination against these viruses should not only prevent disease but lower the incidence of the cancers associated with them. A vaccine against hepatitis B is available as are two vaccines (Gardasil® and Cervarix®) against the most dangerous papilloma viruses.

Are Cancers Contagious?

The short answer is **NO**.

The reason: Cancer cells, like all cells in the body, express histocompatibility molecules on their surface. So like any organ or tissue transplant between two people (other than identical twins), they are **allografts** and are recognized and destroyed by the recipient's immune system.

However, there are some exceptions.

1. Although tumors are not transmissible, viruses are. So any of the viruses described in the previous section can be spread

from person to person and predispose them to the relevant cancers.
2. There have been a number of cases where, unbeknownst to the surgeon, an organ (e.g., a kidney) from a donor with melanoma has allowed the growth of the same melanoma in the recipient. Transplant recipients must have their immune system suppressed if the transplant is not to be rejected, but their immunosuppression also prevents their immune system from attacking the melanoma cells. Stopping immune suppression cures the recipient (but also causes loss of the kidney).
3. Canine transmissible venereal tumor (CTVT). This tumor spreads from dog to dog during copulation. Although the MHC alleles on the tumor cells are only weakly expressed, they do eventually cause the tumor to be rejected.
4. Devil facial tumor disease (DFTD). The carnivorous Tasmanian devil is a marsupial living in Tasmania, Australia. The population is threatened by a facial cancer that is spread through bites. The population is highly inbred, thus closely-related genetically, and the MHC alleles on the tumor are only weakly expressed. So it may be these factors that allow the tumor to grow unchecked.
5. The soft-shell clam, *Mya arenaria*, along the North Atlantic coast of North America is being devastated by a leukemia that spreads from animal to animal perhaps as these filter feeders ingest sea water in which leukemic cells have been shed. These mollusks are invertebrates and lack powerful tissue rejection molecules like the MHC of vertebrates.
6. There are extremely rare cases where a pregnant woman with cancer (a leukemia or melanoma) has transmitted the cancer across the placenta to her fetus (whose immune system has yet to develop).

The Hallmarks of Cancer

In the year 2000 Douglas Hanahan and Robert Weinberg published a paper — The Hallmarks of Cancer — outlining 6 characteristics that are acquired as a cell progresses toward becoming a full-blown cancer. In the 4 March 2011 issue of **Cell**, they add 4 other features.

1. Uncontrolled proliferation.
2. Evasion of growth suppressors. Among the many mutations found in cancers, one or more inactivate **tumor suppressor genes**.
3. Resistance to **apoptosis** (programmed cell death).
4. Develop replicative immortality; i.e., avoid the normal process of cell senescence.
5. Induce angiogenesis; that is, promote the development of a blood supply.
6. Invasion and metastasis — the ability of tumor cells to invade underlying tissue and then to be carried to other parts of the body where secondary tumors develop (metastasis). During this process, the normal adhesion of cells to each other and to the underlying extracellular matrix (ECM) are disrupted.
7. Genomic instability. Cancer cells develop chromosomal aberrations and many (hundreds) of mutations. Most of the latter are "passenger" mutations, but as many as 10 may be "drivers" of the cancerous transformation.
8. Inflammation. Tumors are invaded by cells of the immune system, which promote inflammation. One effect of inflammation is the production of reactive oxygen species (ROS). These damage DNA and other molecules.
9. Changed energy metabolism. Even if well-supplied with oxygen, cancer cells get most of their ATP from glycolysis not cellular respiration.
10. Evade the immune system.

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12.2: Cancer Cells in Culture

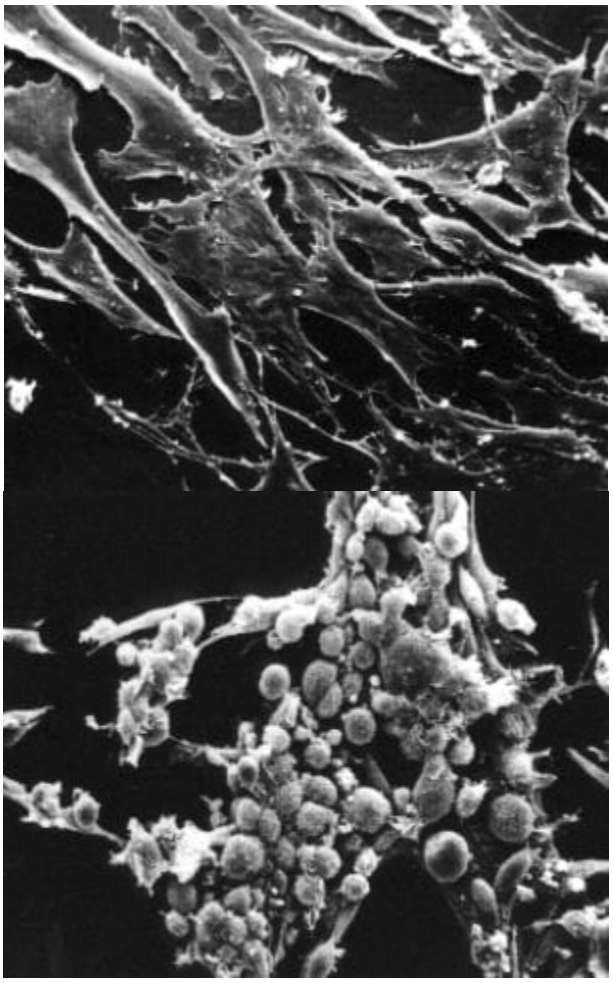


Figure 12.2.2 Cells not showing contact inhibition

The photographs (courtesy of G. Steven Martin) show mouse **fibroblasts** (connective tissue cells) growing in culture. The cells in the top photo show contact inhibition. Those below do not. The cells below are said to be **transformed**. These cells (called 3T3 cells) were not derived from a mouse cancer but were produced by laboratory treatment of normal cells. Radiation, certain chemicals, and certain viruses are capable of transforming cells. Although transformed cells are not derived from cancers, they can often develop into malignant tumors when injected into an appropriate test animal (like a nude mouse).

Normal cells are exceedingly fussy about the nutrients that must be supplied to them in their tissue culture medium.

Cancer cells (and transformed cells) can usually grow on much simpler culture medium.

Normal cells ordinarily have the normal set of chromosomes of the species; that is, have a normal karyotype.

Cancer cells almost always have an abnormal karyotype with

- abnormal numbers of chromosomes (polyploid or aneuploid)
- chromosomes with abnormal structure:
  - translocations
  - deletions
  - duplications
  - inversions

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12.3: Oncogenes

An oncogene is a gene that when mutated or expressed at abnormally-high levels contributes to converting a normal cell into a cancer cell. Cancer cells are cells that are engaged in uncontrolled mitosis.

The signals for normal mitosis

Normal cells growing in culture will not divide unless they are stimulated by one or more growth factors present in the culture medium (e.g, Epidermal Growth Factor (EGF)). The growth factor binds to its receptor, an integral membrane protein embedded in the plasma membrane with its ligand-binding site exposed at the surface of the cell. Examples:

- the Epidermal Growth Factor Receptor (EGFR). The gene encoding it, *EGFR*, is also known as *HER1*.
- another growth factor receptor is encoded by the gene *ERBB2* (also known as *HER2*.)
- Binding of a growth factor to its receptor triggers a cascade of signaling events within the cytosol. Many of these involve
    - kinases — enzymes that attach phosphate groups to other proteins. Examples: the proteins encoded by *SRC*, *RAF*, *ABL*, and the fusion protein encoded by *BCR/ABL* found in chronic myelogenous leukemia (CML).
    - or molecules that turn on kinases. Example: RAS. RAS molecules reside on the inner surface of the plasma membrane where they serve to link receptor activation to "downstream" kinases like RAF.
- In most cases, phosphorylation activates the protein and eventually transfers the signal into the nucleus.

Here phosphorylation activates transcription factors that bind to promoters and enhancers in DNA, turning on their associated genes. Examples: AP-1, a heterodimer of the proteins encoded by *jun* and *fos*. Some of the genes turned on by these transcription factors encode other transcription factors (e.g., *myc*).

Some of the genes turned on by these downstream transcription factors encode cyclins that prepare the cell to undergo mitosis. Genes that participate in any one of the steps above can become oncogenes if they become mutated so that their product becomes constitutively active (that is, active all the time even in the absence of a positive signal) or they produce their product in excess. Possible causes include if their promoter and/or enhancer has become mutated (e.g., the oncomouse: a transgenic mouse that has both copies of its *myc* gene under the influence of extra-powerful promoters) or loss (e.g., by a translocation) of the 3'-UTR of their mRNA so that a microRNA (miRNA) that normally represses translation can no longer do so.

All these oncogenes act as dominants; if the cell has one normal gene (called a proto-oncogene) and one mutated gene (the oncogene) at a pair of loci, the abnormal product takes control. No single oncogene can, by itself, cause cancer. It can, however, increase the rate of mitosis of the cell in which it finds itself. Dividing cells are at increased risk of acquiring mutations, so a clone of actively dividing cells can yield subclones of cells with a second, third, etc. oncogene. When a clone loses all control over its mitosis, it is well on its way to developing into a cancer.

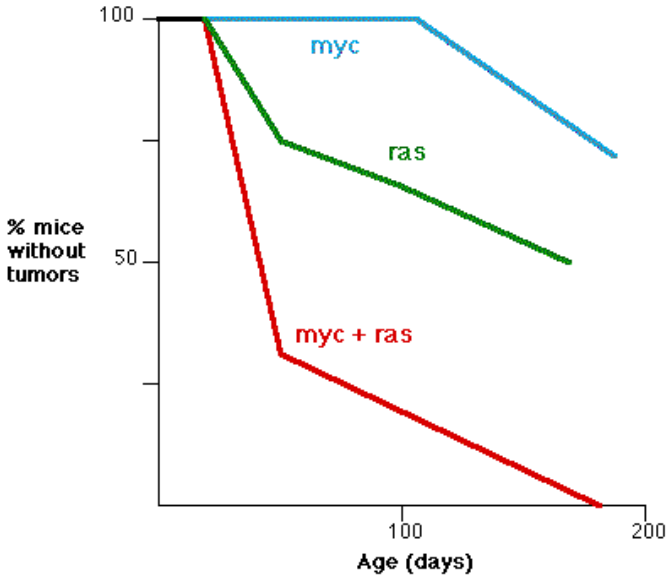


Figure 12.3.1 Synergistic effect of two Oncogenes. Three groups are shown: those mice transgenic for a hyperactive myc alone (blue), those transgenic for ras alone (green), those transgenic for both myc and ras (red)

This graph (based on the work of E. Sinn et al, Cell 49:465,1987) shows the synergistic effect of two oncogenes. The fraction (%) of transgenic mice without tumors is shown as a function of age.

Other types of potential cancer-promoting genes

- **Genes that inhibit apoptosis:** The suicide of damaged cells — apoptosis — provides an important mechanism for ridding the body of cells that could go on to form a cancer. It is not surprising then that inhibiting apoptosis can promote the formation of a cancer. Example: *Bcl-2*. The product of this gene inhibits apoptosis. Overexpression of the gene is a hallmark of B-cell cancers.
- **Genes involved in repairing DNA or stopping mitosis if they fail:** Mutations arise from an unrepaired error in DNA. So any gene whose product participates in DNA repair probably can also behave as an oncogene when mutated. For example: *ATM*. *ATM* (="ataxia telangiectasia mutated") gets its name from a human disease of that name, whose patients — among other things — are at increased risk of cancer. The ATM protein is also involved in detecting DNA damage and interrupting the cell cycle when damage is found. It is estimated that fully 1% of the ~21,000 genes in the human genome are proto-oncogenes.
- **Tumor-Suppressor Genes:** The products of some genes inhibit mitosis. These genes are called tumor suppressor genes. In contrast to oncogenes, these behave as recessives — both alleles must be defective to lose their braking effect on mitosis.

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12.4: Tumor Suppressor Genes

Some genes suppress tumor formation.

- Their protein product inhibits mitosis.
- When mutated, the mutant allele behaves as a **recessive**; that is, as long as the cell contains one normal allele, tumor suppression continues. (Oncogenes, by contrast, behave as dominants; one mutant, or overly-active, allele can predispose the cell to tumor formation).

RB - the retinoblastoma gene

Retinoblastoma is a cancerous tumor of the retina. It occurs in two forms:

- **Familial retinoblastoma**: Multiple tumors in the retinas of both eyes occurring in the first weeks of infancy.
- **Sporadic retinoblastoma**: A single tumor appears in one eye sometime in early childhood before the retina is fully developed and mitosis in it ceases.

Familial retinoblastoma

Familial retinoblastoma occurs when a baby inherits from one of its parents a chromosome (number 13) that has its *RB* locus deleted (or otherwise mutated). The normal Rb protein controls the cell cycle. It integrates the signals reaching the cell to determine whether it is safe for the cell to complete the passage from G1 of the cell cycle to mitosis.

Mechanism

The unphosphorylated Rb protein prevents cells from entering S phase of the cell cycle. It does this by binding to **transcription factors** called **E2F**. This prevents the E2Fs from binding to the promoters of such proto-oncogenes as **c-*myc*** and **c-*fos***. Transcription of c-*myc* and c-*fos* is needed for mitosis so blocking the transcription factors needed to turn on these genes prevents cell division. However, if conditions are adequate for the cell to successfully complete mitosis, the Rb protein becomes phosphorylated, releases the E2Fs, and the cell can proceed through the cell cycle.

The Rb protein also plays a role in mitosis itself: it is needed for proper chromosome condensation starting in prophase, as well as their proper attachment to the spindle. Failure of Rb function during mitosis can lead to aneuploidy and chromosome breakage.

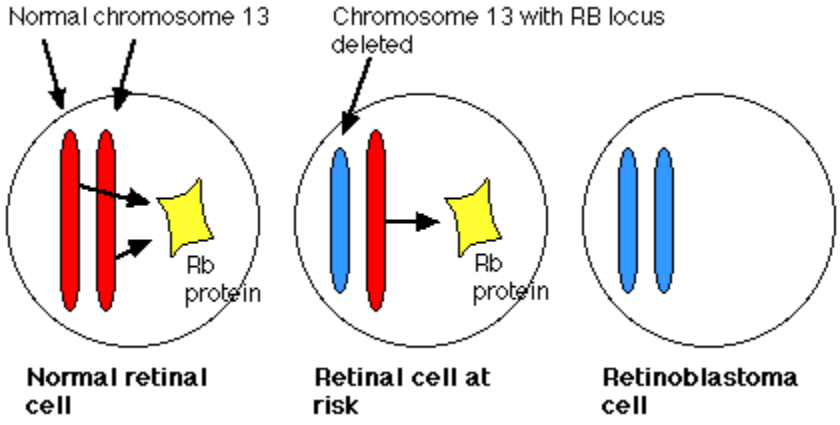


Figure 12.4.1 RB schematic

A random mutation of the remaining *RB* locus in **any** retinal cell — which are nondividing cells and should not enter the cell cycle — completely removes the inhibition provided by the **Rb protein**, and the affected cell grows into a tumor. So, in this form of the disease, a **germline** mutation plus a **somatic** mutation of the second allele leads to the disease.

Sporadic retinoblastoma

In this disease, both inherited *RB* genes are normal and a single cell must be so unlucky as to suffer a somatic mutation (often a deletion) in **both** in order to develop into a tumor. Such a double hit is an exceedingly improbable event, and so only rarely will such a tumor occur. (In both forms of the disease, the patient's life can be saved if the tumor(s) is detected soon enough and the affected eye(s) removed.)

p53

The product of the tumor suppressor gene ***p53*** is a protein of 53 kilodaltons (hence the name). (You will find that the human gene is variously designated as *P53*, *TP53* ["tumor protein 53"], and *TRP53* ["transformation-related protein 53"])

The p53 protein prevents a cell from completing the cell cycle if its DNA is damaged or the cell has suffered other types of damage.

When

- the damage is minor, p53 halts the cell cycle — hence cell division — until the damage is repaired.
- the damage is major and cannot be repaired, p53 triggers the cell to commit suicide by apoptosis.

These functions make *p53* a key player in protecting us against cancer; that is, it is an important tumor suppressor gene. More than half of all human lung, ovarian, and colorectal cancers harbor *p53* mutations and have no functioning p53 protein.

Mice have been cured of cancer by treating them with a peptide that turns on production of the p53 protein in the tumor cells. However, there may be a tradeoff involved: excess production of the p53 protein leads to accelerated aging in mice.

Elephants are very long-lived but seldom develop cancers. It turns out that their cells contain 40 copies of the *p53* gene (*TP53*) compared with the two that we and other mammals have.

$p16^{INK4a}$

The product of the tumor suppressor gene *INK4a* is a protein of 16 kilodaltons (hence the name).

Like p53, it blocks progression through the cell cycle — in this case by inhibiting the action of the cyclin-dependent kinase Cdk4.

As an animal ages, its cells produce increasing amounts of $p16^{INK4a}$. This is probably a good thing in that it reduces the risk of the cell entering uncontrolled mitosis, i.e., becoming a cancer. However, again like p53, there is a tradeoff. As levels of $p16^{INK4a}$ rise in adult stem cells and progenitor cells, their ability to reproduce and thus replace lost or damaged tissue diminishes.

$p16^{INK4a}$ is not simply a reflection of an aging cell but is actively involved in the process.

- Mice expressing higher-than-normal levels of $p16^{INK4a}$ show earlier replicative senescence while
- mice in which $p16^{INK4a}$ activity is blocked continue to repair damaged tissue efficiently but run a higher risk of getting cancer.
- In mice, eliminating senescent cells (they are high in $p16^{INK4a}$) prevents (in young mice) and partially reverses (in older mice) some of the signs of aging such as cataracts, and loss of adipose tissue and skeletal muscle mass.

In humans, deletions and other mutations of $p16^{INK4a}$ are found in a variety of cancers.

Loss Of Heterozygosity (LOH)

Because tumor suppressor genes are recessive, cells that contain one normal and one mutated gene — that is, are heterozygous — still behave normally. (Exception: one X-linked tumor suppressor gene [*WTX*] has been found. In males, having only one X chromosome, a damaging point-mutation in *WTX* or its deletion is all that is needed to eliminate tumor-suppression. Females

are also at risk if the mutation or deletion occurs on the X chromosome that is not inactivated.)

However, there are several mechanisms which can cause a cell to lose its normal gene and thus be predisposed to develop into a tumor. These may result in a "loss of heterozygosity" or "LOH".

Mechanisms of LOH:

1. Deletion of
   - the normal allele;
   - the chromosome arm containing the normal allele;
   - the entire chromosome containing the normal allele (resulting in aneuploidy).
2. In females, X-inactivation of the X chromosome carrying the normal allele.
3. Loss of the chromosome containing the normal allele followed by **duplication** of the chromosome containing the mutated allele.
4. Mitotic recombination. The study of tumor suppressor genes revealed (for the first time) that crossing over — with genetic recombination — occasionally occurs in mitosis (as it always does in meiosis).

In #3 and #4, the resulting cell now carries two copies of the "bad" gene. This is called "reduction to homozygosity".

LOH can work both ways.

When LOH occurs by mitotic recombination (process #4 above), one daughter cell becomes homozygous for the mutant allele but the other becomes homozygous for the normal ("wild-type") allele. This is of no help when tumor-suppressor genes are involved but in other situations it can be.

A rare skin disease of humans called **ichthyosis with confetti** is an example. It is caused by the inheritance of a dominant mutation in one of the keratins (which make intermediate filaments). At birth, infants with the disease have uniformly reddened skin whose cells are heterozygous for the dominant mutant allele and a normal keratin allele. But as the years go by, an increasing number of patches of normal, whitened, skin appear (like "confetti"). Genomic analysis reveals that each patch develops from a skin stem cell that by mitotic recombination has undergone "reduction to homozygosity". In this case, the daughter cell that inherits two normal keratin alleles goes on to generate a patch of normal skin. This work is described in Choate, K.A., *et al.*, *Science* **330**:94-97 (1 October 2010).

Mutation is not the only way to inactivate tumor suppressor genes.

Their function can also be blocked by methylation of their promoter.

Cancer cells often contain a methylated promoter on one tumor suppressor gene accompanied by

- a similarly blocked promoter on the other allele (producing the same effect as #2 above);
- a loss of that locus on the other chromosome (like the LOH in #1 above);
- an inactivating mutation in the other allele.

Tumor suppressor genes = anti-oncogenes

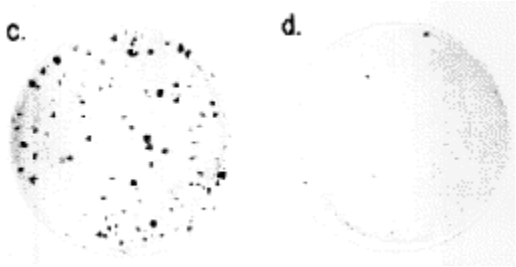


Figure 12.4.2 Cell Colonies

Genes like ***RB*** and ***p53*** are also called anti-oncogenes. They were first given this name because they reverse, at least in cell culture, the action of known oncogenes. This image (courtesy of Moshe Oren, from *Cell* **62**:671, 1990) shows petri dishes which

were seeded with the same number of mouse cells that had been transformed by **two oncogenes**: ***myc*** and ***ras***. Many of those on the left have grown into colonies of cells. However, the cells plated on the right also contained the tumor suppressor ***p53*** gene. Only a few have been able to grow into colonies.

Human Papilloma Viruses (HPV)

The name anti-oncogene may be even more appropriate than originally thought. Both the Rb protein and the p53 protein turn out to complex directly in the cell with a gene product of some human papilloma viruses.

Once inside the cells of their host, these viruses synthesize a protein designated **E7** and another designated **E6**.

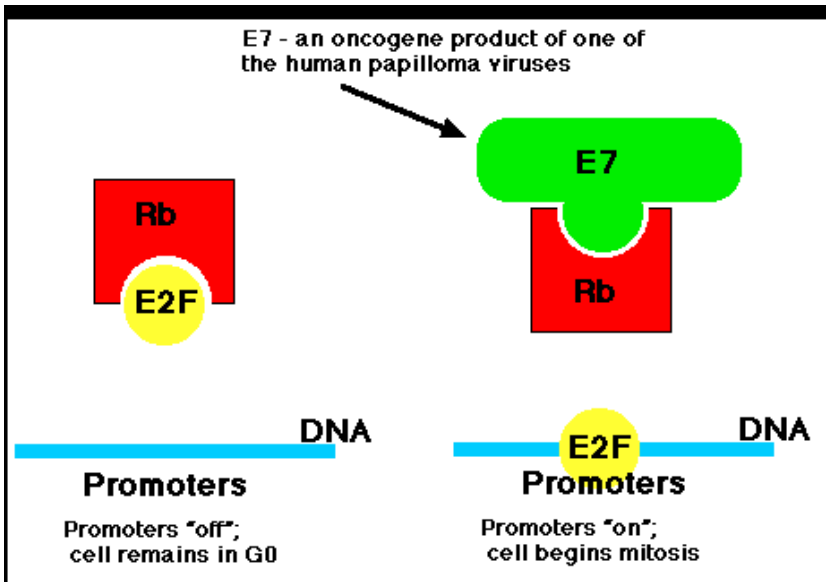


Figure 12.4.3 HPV

Of the >30 strains of HPV that infect humans, several, especially HVP-16 and HPV-18, have been implicated as a risk factor for **cervical cancer** and also cancers of the throat. Their **E7** protein binds to the **Rb** protein preventing it from binding to the host transcription factor **E2F**.

Result: E2F is now free to bind to the promoters of genes (like **c-*myc***) that cause the cell to enter the cell cycle (right). Thus this version of E7 is an **oncogene product**.

The E6 protein binds the **p53** protein targeting it for destruction by proteasomes and thus removing the block on the host cell's entering the cell cycle.

Although the figure shows the "off" promoters as empty, it is now clear that being "off" involves both

- the absence of activators of transcription **and**
- the presence of repressors of transcription.

A cell cannot remain in G0 of the cell cycle without these repressors. Perhaps mutant versions of them are another cause of cancer (cancer cells are never in G0).

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12.5: BCL-2

BCL-2 is a human proto-oncogene located on chromosome 18. Its product is an integral membrane protein (called Bcl-2) located in the membranes of the endoplasmic reticulum (ER), nuclear envelope, and in the outer membranes of mitochondria. The gene was discovered as the translocated locus in a **B**-**c**ell **l**eukemia (hence the name). This translocation is also found in some B-cell lymphomas.

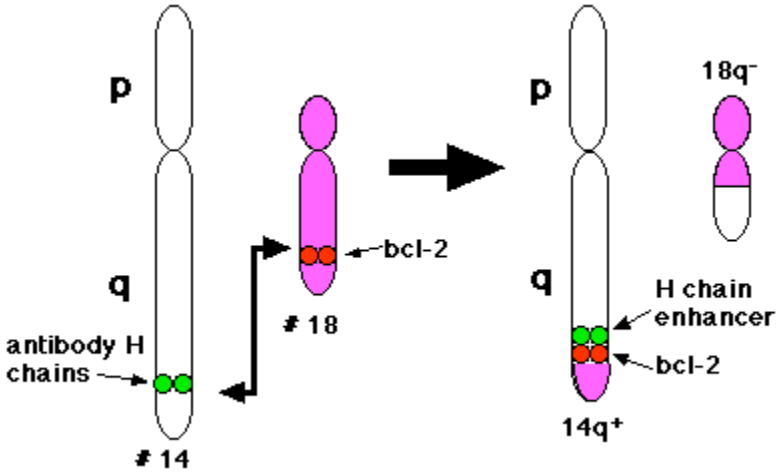


Figure 12.5.1: *BCL - 2*

In the cancerous B cells, the portion of chromosome 18 containing the *BCL-2* locus has undergone a reciprocal translocation with the portion of chromosome 14 containing the antibody heavy chain locus. This t(14;18) translocation places the *BCL-2* gene close to the heavy chain gene enhancer. This enhancer is very active in B cells (whose job it is to synthesize large amounts of antibody). So it is not surprising to find that the Bcl-2 protein is expressed at high levels in these t(14;18) cells.

What makes *BCL-2* a proto-oncogene?

B cells, like all activated lymphocytes, die a few days after they have had a chance to do their job. This ensures that they do not linger around after the threat has been dealt with and turn their attack against self components. Aging B cells kill themselves by **apoptosis**. However, high levels of the Bcl-2 protein protect the cells from early death by apoptosis. The Bcl-2 protein suppresses apoptosis by preventing the activation of the caspases that carry out the process. So genes encoding inhibitors of apoptosis must be added to the list of genes that can act as oncogenes. In this case the effect is not achieved by increasing the rate of cell proliferation but by reducing the rate of cell death.

Although the t(14:18) translocation is found in B-cell lymphomas and leukemias, something else must contribute to creating the cancer because over 50% of us have small numbers of B-cells with that translocation that never progress to cancer. The antibody gene loci are dangerous places for proto-oncogenes to take up residence. Translocation of the proto-oncogene **c-myc** close to the enhancer of the antibody heavy chain genes also produces cancerous B cells resulting in Burkitt's lymphoma. The translocation of the *BCL-2* locus is just one of many mutations that can give rise to a malignant clone of B cells. All of the resulting leukemias are designated **chronic lymphocytic leukemia** or **CLL**.

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12.6: Burkitt's Lymphoma

Burkitt's lymphoma is a solid tumor of **B lymphocytes**, the lymphocytes that the immune system uses to make **antibodies**. The genes for making antibodies are located on chromosomes **14** (the heavy [H] chains), **2** (kappa light chains), and **22** (lambda light chains). These genes are expressed only in B lymphocytes because only B cells have the necessary transcription factors for the promoters and enhancers needed to turn these antibody genes "**on**". In most (approximately 90%) of the cases of Burkitt's lymphoma, a reciprocal translocation (designated t(8;14) has moved the **proto-oncogene c-*myc*** from its normal position on chromosome **8** to a location close to the enhancers of the antibody heavy chain genes on chromosome **14**.

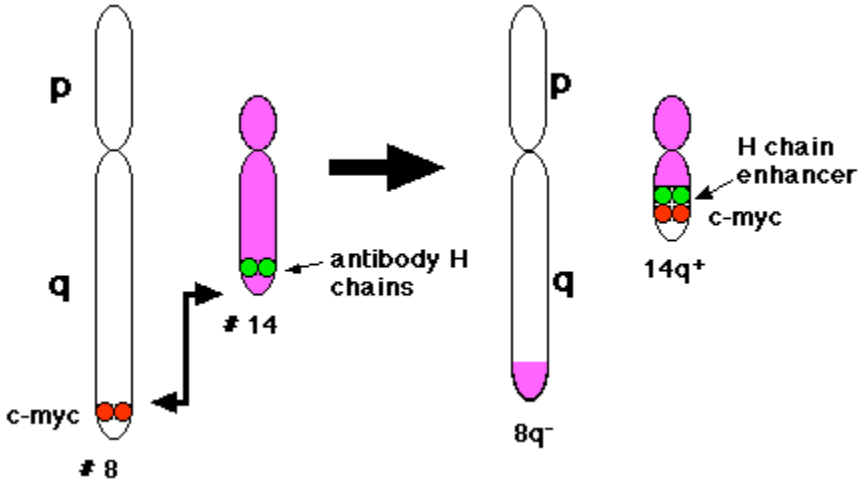


Figure 12.6.1: *Chromosome 14*

In all the other cases, c-*myc* has been translocated close to the antibody genes on chromosome 2 or 22. In every case, c-*myc* now finds itself in a region of vigorous gene transcription, and it may simply be the overproduction of the c-myc product (a transcription factor essential for mitosis of mammalian cells) that turns the lymphocyte cancerous. Uncontrolled mitosis of this cell results in a clone of cancer cells, Burkitt's lymphoma. Many other human cancers involve chromosome aberrations, such as translocations, at the loci of known proto-oncogenes.

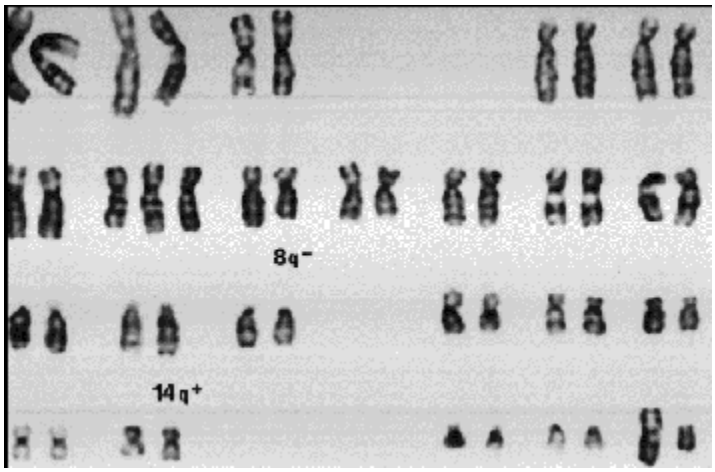


Figure 12.6.2: *Karyotype of a cell with Burkitt's lymphoma*

Figure 12.6.2 is an actual karyotype (courtesy of Janet Finan and C. M. Croce) of a cell from the tumor of a patient with Burkitt's lymphoma. The long (q) arm of the resulting chromosome 8 is shorter ($8q^-$) than its normal homologue; the long arm of translocated chromosome 14 longer ($14q^+$). The heavy chain gene locus on chromosome 14 is a dangerous place. Several other proto-oncogenes produce cancerous B cells — leukemias, lymphomas, and multiple myelomas — when translocated into this locus. The risk of translocations involving the heavy chain gene locus is probably especially high because breaks in its DNA occur

naturally during the synthesis of antibodies.

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12.7: Chronic Myelogenous Leukemia (CML)

Leukemia is an uncontrolled proliferation of one kind of white blood cell (or **leukocyte**). Like all cancers (probably), all the leukemic cells are descended from a **single cell** that lost the ability to maintain normal control over the cell cycle. There are a number of types of leukemia, as you would expect from the number of types of white blood cells (5) and the number of stages they pass through as they mature. One of the most common is **c**hronic **m**yelogenous **l**eukemia or **CML**.

Chronic Myelogenous Leukemia (CML) arises in a bone marrow **stem cell** that is the precursor to all the types of blood cells. However, it usually affects the so-called myeloid lineage (hence the name) that produces granulocytes and macrophages. As the name suggests, the disease often exists for years with only moderately elevated numbers of leukemic cells (descended from the stem cells) and few symptoms. At some point, however, the patient goes through a "blast crisis" when the leukemic granulocyte-macrophage progenitors begin to divide by themselves — increasing their numbers enormously while failing to continue their differentiation.

The Philadelphia Chromosome (Ph^1)

In most cases of CML, the leukemic cells share a chromosome abnormality not found in any nonleukemic white blood cells, nor in any other cells of the patient's body. This abnormality is a reciprocal translocation between one chromosome **9** and one chromosome **22**. This translocation is designated **t(9;22)**. It results in one chromosome 9 longer than normal and one chromosome 22 shorter than normal. The latter is called the **Philadelphia chromosome** and designated Ph^1.

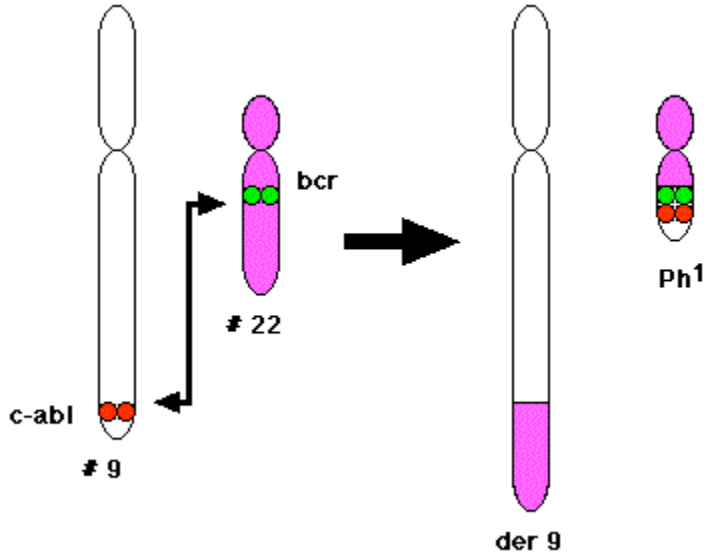


Figure 12.7.1: Translocation between chromosome 9 and 22. Reciprical translations between one # 9 and one #22 chromosomes forms an extra long chromosome ("der 9") and the Philadelphia chromosome (Ph^1) containting the fused abl-bcr gene. This is a schematic view representating metaphase chromosomes.

The DNA removed from chromosome 9 contains most of the **proto-oncogene** designated **c-ABL**. The break in chromosome 22 occurs in the middle of a gene designated **BCR**. The resulting Philadelphia chromosome has the 5' section of BCR fused with most of c-ABL.

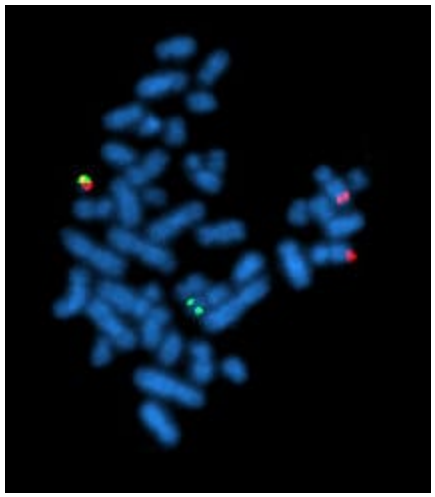


Figure 12.7.1: *FISH image of bcr/abl positive rearranged metaphase (CC-SA-BY-3.0);*

The micrograph in Figure 12.7.2 uses **f**luorescence **i**n **s**itu **h**ybridization (FISH) to reveal the **ABL** DNA (red) and the **BCR** DNA (green) in the interphase nuclei of the leukemic cells of a patient with CML. The red dot at left center reveals the location of ABL on the normal chromosome 9; the green dot (top center) shows BCR on the normal chromosome 22. The combined dots (red + green = yellow) at the lower right reveal the fused **BCR-ABL** gene on the Philadelphia chromosome. Figure 12.7.3 is a schematic which can help you interpret the micrograph.

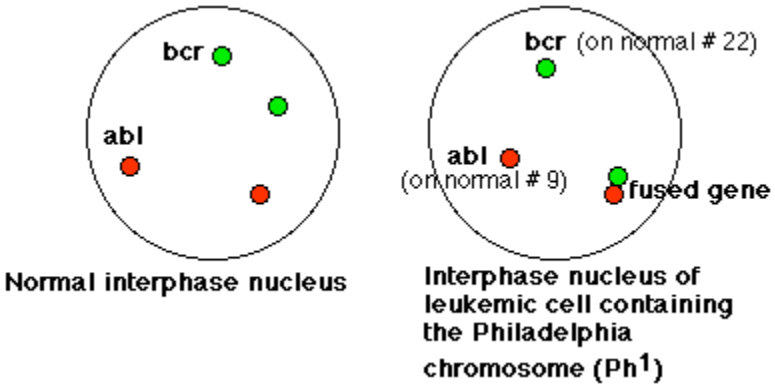


Figure 12.7.3: *Schematic of ABL DNA*

Transcription and translation of the hybrid **BCR-ABL** gene produces an abnormal ("fusion") protein that activates constitutively (all the time) a number of cell activities that normally are turned on only when the cell is stimulated by a growth factor, such as platelet-derived growth factor (**PDGF**).

This unrestrained activation increases the rate of mitosis and protects the cell from apoptosis. The outcome is an increase in the number of Ph^1-containing cells. During the chronic phase of the disease, these are still able to exit the cell cycle and to differentiate into mature cells that perform their normal functions. At some point, however, another mutation in a proto-oncogene (**RAS**, for example) or in a **tumor-suppressor gene** (**p53**, for example), will occur in one of these cells. The additional mutation causes the rate of mitosis in that cell and its descendants to rise sharply. The daughter cells fail to differentiate and the patient enters the crisis phase of the disease.

A Promising Treatment

Until recently, the only successful treatment of CML was to destroy the patient's bone marrow and then restore blood-cell production by infusing stem cells from the bone marrow of a healthy donor. But now treatment with the drug imatinib mesylate (**Gleevec®** also known STI571) appears to be able to cure the disease. This molecule fits into the active site of the

ABL protein preventing ATP from binding there. Without ATP as a phosphate donor, the ABL protein cannot phosphorylate its substrate(s). A phase 2 study, found that almost 90% of the CML patients treated with the drug showed no further progression of their disease.

Gleevec also shows promise against one type of stomach cancer (gastrointestinal stromal tumors = GIST), which is a life-threatening excessive production of eosinophils. In this disease, Gleevec inhibits a different overactive tyrosine kinase. This one also results from the fusion of parts two different genes (because of the deletion of the DNA between them):

- the first 233 codons of a gene designated *FIP1L1* fused to
- the final 523 codons of the gene (*PDGFRα*) encoding the tyrosine kinase domain of a **receptor** for platelet-derived growth factor. The fusion protein produced, like BCR-ABL, is hyperactive.

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12.8: Fighting Cancer with Inhibitors of Angiogenesis

Once a nest of cancer cells reaches a certain size (1–2 mm in diameter), it must develop a blood supply in order to grow larger. Diffusion is no longer adequate to supply the cells with oxygen and nutrients and to take away wastes. Cancer cells (probably like all tissues) secrete substances that promote the formation of new blood vessels — a process called **angiogenesis**. Over a dozen substances have been identified that promote angiogenesis. A few examples are angiopoietin-1, the basic fibroblast growth factor (**bFGF**) and the vascular endothelial growth factor (**VEGF**).

Curiously, some tumors also secrete substances that **inhibit** angiogenesis. This explains a clinical phenomenon that has been known for decades:

- A patient has a tumor, the so-called **primary tumor**.
- There is no evidence that the primary tumor has metastasized.
- A surgeon removes the primary tumor.
- Some weeks later metastases of the tumor appear throughout the patient's body
- The speed of their appearance indicates that they were present all along, but too small to be detected.

This phenomenon caused Dr. Judah Folkman of Children's Hospital and the Harvard Medical School in Boston to hypothesize that a large primary tumor secretes not only stimulators of its own angiogenesis but angiogenesis **inhibitors** that are released into the circulation and inhibit angiogenesis — and thus further growth — of any metastases of the primary tumor. A number of inhibitors of angiogenesis have been discovered.

Angiostatin

Angiostatin is a polypeptide of approximately 200 amino acids. It is produced by the cleavage of **plasminogen**, a plasma protein that is important for dissolving blood clots. Angiostatin binds to subunits of ATP synthase exposed at the surface of the cell embedded in the plasma membrane. (Before this recent discovery, ATP synthase was known only as a mitochondrial protein.)

Endostatin

Endostatin is a polypeptide of 184 amino acids. It is the globular domain found at the C-terminal of Type XVIII (18) collagen (a collagen found in blood vessels) cut off from the parent molecule.

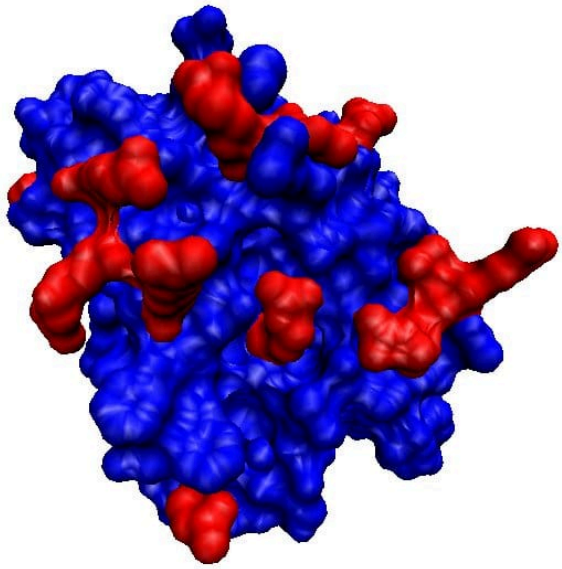


Figure 12.8.1: Endostatin monomer, basic amino acid residues shown in red (source: pdb.org, 1KOE).

Effects of angiostatin and endostatin in mice

Injections of angiostatin inhibit the metastasis of certain (mouse) primary tumors. Injections of endostatin (made by recombinant DNA technology) cause the primary tumor to regress. In time, the primary tumor reappears, but a repeat injection causes it to regress again. Each time it reappears, the tumor is just as susceptible to treatment as before. After a few cycles of growth, treatment, and regression, the primary tumor finally stops growing (at least in the cases examined) and remains dormant at a small size.

Some human tumors can be grown in immunodeficient mice. (Being immunodeficient, they cannot reject this foreign tissue). Treatment with endostatin caused these human tumor masses to shrink in their mouse host. Combined treatment with **both** angiostatin and endostatin has caused some primary mouse tumors to disappear entirely.

This is not seen with conventional chemotherapy. Repeated exposure to chemotherapeutic drugs selects for the appearance of drug-resistant tumor cells. Eventually, further drug treatment is worthless. Why the difference in response? Chemotherapy works directly on tumor cells which mutate easily. Angiogenesis inhibitors don't work on the tumor cells but on normal cells involved in the formation of blood vessels.

Other Angiogenesis Inhibitors

Epithelial cells express transmembrane proteins on their surface — called **integrins** — by which they anchor themselves to the extracellular matrix. It turns out that the new blood vessels in tumors express a **vascular integrin** — designated **alpha-v/beta-3** — that is not found on the old blood vessels of normal tissues.

Vitaxin®, a humanized monoclonal antibody directed against the alpha-v/beta-3 vascular integrin, shrinks tumors in mice without harming them. In Phase II clinical trials in humans, Vitaxin has shown some promise in shrinking solid tumors without harmful side effects.

What does the future hold for angiogenesis inhibitors?

Clinical trials of endostatin (manufactured by recombinant DNA technology), in combination with standard chemotherapy have shown some benefit in one type of lung cancer.

Bevacizumab (Avastin®). This is a humanized monoclonal antibody that binds to VEGF thus keeping it from binding to its receptors. Approved by the US FDA in February 2004 for the treatment of colorectal cancers.

Ranibizumab (Lucentis®) is a modified version of Avastin® that is showing great promise in inhibiting the formation of new blood vessels in the retina — the cause "wet" macular degeneration.

Trials are also scheduled to begin on a **synthetic ribozyme** that blocks synthesis of the VEGF receptor. These are only a few examples of the ~50 antiangiogenesis drugs now in clinical trials.

But proceed with caution.

In animal studies, some cancers — notably pancreatic cancer — have turned out to resist chemotherapy because of their poor blood supply. Perhaps such cancers need to have angiogenesis promoted; inhibiting it could make a bad problem worse.

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12.9: Immunotherapy of Cancer

Most cancer patients are treated with some combination of surgery, radiation, and chemotherapy. Radiation and chemotherapy have the disadvantage of destroying healthy as well as malignant cells and thus can cause severe side-effects. What is needed are more precisely-targeted therapies. One long-held dream is that the specificity of immune mechanisms could be harnessed against tumor cells. This might use the patient's own immune system or the transfer of antibodies or T cells from an outside source (i.e. passive immunization). Ideally, these agents would be targeted to molecules expressed on the cancer cells but not on healthy cells. However, such **tumor-specific antigens** have been hard to find, and so many of the immune agents now in use do target healthy cells as well.

Immunostimulants

There is considerable evidence that cancer patients have T cells that are capable of attacking their tumor cells. In fact, it may be that the appearance of cancer is a failure of **immune surveillance**: the ability of one's own immune system to destroy cancer cells as soon as they appear. But what to do if they fail? Immunostimulants are nonspecific agents that tune-up the body's immune defenses. There have been some successes with

- injecting adjuvant-like agents directly into the tumor. The only one that succeeds often enough to remain in use is the bacterial preparation BCG. Introduced into the bladder, it can help eradicate early-stage bladder tumors.
- Oral therapy with levamisole, a drug widely-used for deworming (people as well as animals), has been used to treat a variety of cancers but with inconsistent results.
- interleukin-2 (IL-2), a potent growth factor for T cells;
- alpha-interferon (IFN-α)

Cancer Therapy with Monoclonal Antibodies

A number of monoclonal antibodies show promise against cancer, especially cancers of white blood cells (leukemias, lymphomas, and multiple myeloma). Some examples:

- **Rituximab** (trade name = Rituxan®). Used to treat B-cell lymphomas. The CD20 molecule to which it binds is present on most B-cells, healthy as well as malignant, but over the months following treatment, new healthy B cells are formed from precursors that do not have CD20 and thus were not destroyed by the treatment.
- **Trastuzumab** (trade name = Herceptin®). Binds HER2, a growth factor receptor found on some tumor cells (some breast cancers, lymphomas). The only monoclonal so far that seems to be effective against solid tumors.
- **Alemtuzumab** (MabCampath®). Binds to CD52, a molecule found on white blood cells. Has produced remission of chronic lymphocytic leukemia.
- **Lym-1** (Oncolym®). Binds to the HLA-DR-encoded histocompatibility antigen that can be expressed at high levels on lymphoma cells.
- **Bevacizumab** (Avastin®). Binds to vascular endothelial growth factor (VEGF) thus blocking its action and depriving the tumor of its blood supply.
- **Cetuximab** (Erbitux®). Used to treat colorectal cancers.
- A monoclonal antibody against CD47. CD47 is a cell-surface protein expressed at high levels in many different human cancers. CD47 blocks any effort that macrophages and dendritic cells might make to phagocytose the cancer cells; that is, CD47 is a "don't eat me" signal. A variety of human cancers transplanted into immunodeficient mice have their growth suppressed and metastases prevented when the mice are given a monoclonal antibody against CD47 thus unleashing the ability of phagocytes to destroy the cancer cells. The success in mice will soon lead to clinical trials in humans.
- **Ipilimumab** (Yervoy®). Unlike the other monoclonals listed here, ipilimumab acts as an **immunostimulant**. It does so by binding to the CTLA-4 molecules on the T cell so that they cannot bind to the B7 molecules on the antigen-presenting cell. This frees the T cell's CD28 molecules to bind B7 thus receiving the stimulatory "signal 2" from the antigen-presenting cell. Ipilimumab was approved by the U.S. Food and Drug Administration on 25 March 2011 for use against metastatic melanoma. Because its double-negative effect works to enhance the body's overall T-cell responses, it may well turn out to be useful against other cancers as well (and explains some of the autoimmune-like side effects it produces). It also provides perhaps the best evidence yet for the existence of immune surveillance; that is, the presence in the patient's body of an innate population of T cells specific for the tumor.

- **Pembrolizumab** and **nivolumab**

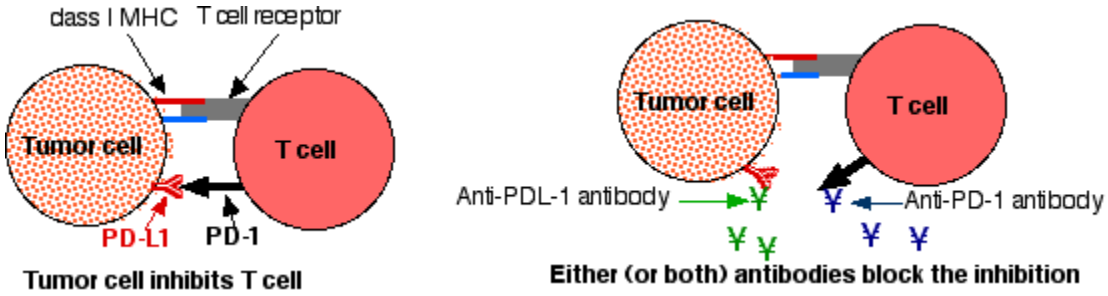


Figure 12.9.1: Tumor cell Inhibits and Antibodies

- **Blinatumomab** is a synthetic monoclonal antibody each arm of which carries a binding site with a different specificity:
  - one arm binds to CD19, an antigen found on the surface of B cells and B-cell lymphomas
  - the other arm binds to CD3, a cell-surface molecule on T cells, including cytotoxic T lymphocytes (CTLs)

By forming a bridge between CD3 and CD19, blinatumomab is able to attach T cells to B cells and activate the T cells to kill the B cells. Early clinical trials with blinatumomab on a small number of patients appear quite promising. Modest doses of the drug produced partial and, in a few cases, complete regression of their lymphoma.

Immunotoxins

A major problem with chemotherapy is the damage the drugs cause to all tissues where rapid cell division is going on. What is needed is a "magic bullet", a method of delivering a cytotoxic drug directly and specifically to tumor cells, sparing healthy cells. Such a magic bullet would have two parts - a monoclonal antibody specific for the cancer cell attached to a cytotoxic drug or toxin that kills the cell once it gets inside.

Some two dozen immunotoxins are in clinical trials. Two that have already received FDA approval:

1. **Adcetris®.** The vedotin is attached to the monoclonal antibody by a bridge that is cleaved once the conjugate is safely inside the tumor cell releasing the toxin to do its work there. In one trial, 73% of the patients with Hodgkin's lymphoma went into remission.
   - a monoclonal antibody that binds **CD30**, a cell-surface molecule expressed by the cells of some lymphomas but not found on the normal stem cells needed to repopulate the bone marrow.
   - **vedotin**, a drug that blocks mitosis by preventing the polymerization of tubulin (needed to form the mitotic spindle).
2. **Kadcyla®** The DM1 is attached to the monoclonal antibody by a bridge that is cleaved once the conjugate is safely inside the tumor cell releasing the toxin to do its work there. Kadcyla® prolongs survival in women whose breast cancer over-expresses HER2 (about 20% of breast cancer cases).
   - Trastuzumab (Herceptin®), the monoclonal antibody against HER2 listed above;
   - DM1, another drug that inhibits mitosis by preventing the polymerization of tubulin (needed to form the mitotic spindle).

Radioimmunotherapy

Monoclonal antibodies against tumor antigens can also be coupled to radioactive atoms. The goal with these agents is to limit the destructive power of radiation to those cells (cancerous) that have been "fingered" by the attached monoclonal antibody. Examples:

- **Zevalin®**. This is a monoclonal antibody against the CD20 molecule on B cells (and lymphomas) conjugated to either
  - the radioactive isotope indium-111 (^{111}In) or
  - the radioactive isotope yttrium-90 (^{90}Y)

  Both are given to the lymphoma patient, the ^{111}In version first followed by the ^{90}Y version (in each case supplemented with Rituxan®).
- **Bexxar®** (tositumomab). This is a conjugate of a monoclonal antibody against CD20 and the radioactive isotope iodine-131 (^{131}I). It, too, is designed as a treatment for lymphoma. Although both Bexxar® and Zevalin® kill normal B cells, they don't

harm the B-cell precursors because these do not express CD20. So, in time, the precursors can repopulate the body with healthy B cells.

On 3 February 2005, the New England Journal of Medicine reported that 59% of patients with a B-cell lymphoma were disease-free 5 years after a single treatment with ^{131}I-tositumomab (a treatment that was relatively free of the nasty side-effects, e.g., hair loss, of conventional chemotherapy).

Adoptive Cell Therapy (ACT)

Tumor destruction is done by cells. Antibodies may help, but only by identifying the cells to be destroyed, e.g., by macrophages. But T cells, e.g., cytotoxic T lymphocytes (CTL), are designed to destroy target cells. What about enlisting them in the fight?

Tumor-Infiltrating Lymphocytes (TIL)

Solid tumors contain lymphocytes that are specific for antigens expressed by the tumor. For many years, Steven A. Rosenberg and his associates at the U. S. National Cancer Institute have tried to enlist these cells in cancer therapy.

On September 19, 2002, he reported his most promising results at that time. The procedure:

- Isolate T cells — both $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T lymphocytes (CTL) from samples of the tumor (melanoma)
- Test them in vitro to find the most efficient killers of the melanoma cells.
- Grow large numbers of them in culture (using the powerful T-cell growth factor IL-2).
- Treat the patient with modest doses of cytotoxic drugs to reduce — but not destroy — the bone marrow (called nonmyeloablative conditioning).
- Reintroduce the mix of Th cells ($CD4^+$) and CTL ($CD8^+$) into the patient (along with IL-2).

The results:

- The infused cells usually took up long-term residence.
- In 10 of 13 patients, their melanoma cells — including all metastases — regressed either partially or completely.

In a few cases, the TIL seemed to be reacting to tumor-specific antigens, but in most the target seems to have been antigens expressed by all melanin-containing cells. Evidence:

- Four patients lost normal melanocytes from their skin leaving white patches.
- One patient developed inflammation of the uvea, the coat of melanin-containing cells within the eye.

Adoptive transfer of a clone of the patient's own tumor-antigen-specific T cells

The 19 June 2008 issue of the New England Journal of Medicine (Naomi Hunder *et al*) carried a report describing the successful treatment of a man with metastasized melanoma using his own T cells. The procedure:

- His leukocytes were harvested and a mixed culture was prepared containing
  - antigen-presenting dendritic cells.
  - a peptide from the antigen **NY-ESO-1**. NY-ESO-1 is a protein that is produced by several types of tumors (e.g., melanoma, lung and breast cancers) but is not expressed by normal cells (except those in the testis).
  - The patient's own T cells.
- After repeated stimulation with the antigen, responding cells were **cloned** by limiting dilution.
- One (of four) antigen-reactive cells was then expanded in culture until
- 5 billion (5×10^9) identical anti-NY-ESO-1 $CD4^+$ T cells were available to infuse into the patient.

The result: complete regression of each metastatic clump of melanoma cells, and the patient has remained free of this lethal cancer for two years since this treatment.

Adoptive transfer of genetically-modified T cells

Genetically engineered with a T-cell Receptor

On April 20, 2006, the Rosenberg group reported some success with melanoma patients using a modification of the TIL procedure.

- The patient's T cells were removed and treated with a retroviral vector containing the αβ T-cell receptor (TCR) specific for a melanoma antigen.
- Large numbers of these were grown in culture.
- After nonmyeloablative conditioning to "make room" for them, the genetically-modified lymphocytes were infused into the patient.
- This application of gene therapy succeeded in eliminating the metastases and providing a disease-free period of two years in two patients.

Genetically engineered with a Chimeric Antigen Receptor (CAR)

The 10 August 2011 online version of the *New England Journal of Medicine* carried a report by Porter, D., *et al.* on their results with one (of three) patients treated for chronic lymphocytic leukemia (CLL) with an infusion of his own genetically-modified T cells.

The patient's malignant B cells expressed the surface antigen CD19 just as normal B cells do.

T cells were harvested from his blood and later treated with a vector encoding the antigen-binding site of an anti-CD19 **antibody** along with two other costimulatory molecules. The result: some 5% of these T cells expressed this synthetic antibody (called a chimeric antigen receptor or CAR) and were activated when they bound CD19 with it (rather than with their T cell receptor (TCR) which they would normally use).

Injected back into the patient, they proliferated by some 1000-fold and persisted for months. During this period, they eliminated all his malignant B cells (as well as his normal B cells). At the time of the report (10 months after treatment), he continued to be free of his cancer. Lacking normal B cells as well, he needed periodic infusions of immune globulin to keep infections at bay.

"One swallow does not make a summer", but these results give hope that in time immunotherapy will become an effective weapon against cancer.

Cancer Vaccines

Any response of the patient's own immune system – immune surveillance – has clearly failed in cancer patients. The purpose of cancer vaccines is to elicit a more powerful active immunity in the patient. Several approaches are being explored.

Patient-Specific Cancer Vaccines

Patient-Specific Dendritic-Cell Vaccines

Dendritic cells are the most potent antigen-presenting cells. They engulf antigen, process it into peptides, and "present" these to T cells.

To make a dendritic-cell vaccine,

- Harvest dendritic cells from the patient.
- Expose these in vitro to antigens associated with the type of tumor in the patient.
  - The antigens are found in normal – as well as cancerous – cells of that tissue (e.g., tyrosinase in melanocytes, prostatic acid phosphatase [PAP] in prostate cells).
  - They may be fused with a stimulatory molecule such as granulocyte-macrophage colony-stimulating factor (GM-CSF)
- Inject these "pulsed" dendritic cells back into the patient.
- Hope that they elicit an strong cell-mediated immune response, e.g. by cytotoxic T lymphocytes (**CTL**).

On 29 April 2010 the U.S. Food and Drug Administration approved the first anti-cancer vaccine: a patient-specific dendritic-cell vaccine for use against advanced prostate cancer. The vaccine, called sipuleucel-T (Provenge®), is produced by pulsing the patient's dendritic cells with a fusion protein coupling prostatic acid phosphatase [PAP] with GM-CSF.

Patient-Specific Tumor-Antigen Vaccines

The antigens in these vaccines are taken from the patient's own tumor cells.

- Harvest some tumor cells from the patient.
- Ship them to a company that will use them to make complexes with adjuvant materials.
- The complexes are returned to be injected into the patient.

Several of such vaccines are currently in clinical trials.

Tumor-Antigen-Specific Vaccines

These vaccines are used to immunize the patient with an antigen universally expressed by tumors of that type (but not by normal cells) mixed with some form of adjuvant that will enhance the response.

Examples:

- Many cancer patients mount an immune response — both antibody-mediated and cell-mediated — against the tumor (and testis) antigen NY-ESO-1. Deliberate immunization with this protein (plus an adjuvant) boosts this response and has shown some promise in early clinical trials. (Cells in the testis do not express HLA antigens, so are not at risk from attack by NY-ESO-1-specific cytotoxic T lymphocytes).
- **MAGE-A3** is another protein common on cancer cells. A vaccine using MAGE-A3 — along with an adjuvant — is in Phase III clinical trials to assess its effectiveness against melanoma and lung cancer.
- HER2 is a protein over-expressed on 20–30% of breast cancers. NeuVax® is a vaccine that contains a peptide of HER2 along with recombinant GM-CSF as an adjuvant. It stimulates the formation of cytotoxic T lymphocytes (CTLs) that attack cells expressing HER2 and has shown promise in clinical trials.

Unlike patient-specific vaccines, these vaccines can be mass-produced for use in anyone with the appropriate tumor.

Combining Procedures #3 and #4

While tumors are immunogenic in the patient who carries them, they are only weakly so. In the hopes of improving cancer immunotherapy, clinical trials are now proceeding to test the efficacy of combining potent patient-specific cancer immunization with treating the patient with large numbers of cultured cancer-antigen-specific T cells that result.

The patient is repeatedly immunized with his or her own cancer cells along with a strong adjuvant (e.g., GM-CSF) followed by harvesting the patient's leukocytes and growing large numbers of them in the laboratory before infusing them into the patient along with interleukin-2. This combined approach — which generates large numbers of patient-cancer-specific killer T cells — has been tested against kidney and one type of brain cancer with promising results.

Blood Cancers

Cancers of blood cells, leukemias and lymphomas, arise in the bone marrow — the source of all blood cells.

One approach to curing leukemia is to treat the patient with such high doses of chemotherapy and radiation that not only are the leukemic cells killed, but the patient's bone marrow is destroyed. If the patient is to survive the treatment, called "myeloablative conditioning", he or she must be given a transplant of hematopoietic stem cells — the cells from which all blood cells are formed.

The stem cells can be

- an **auto**graft; that is, from bone marrow harvested from the patient and stored before treatment begins. In this case, however, the marrow must also be treated to purge it of all cancer cells it may contain before it is returned to the patient. This

sometimes fails.
- an **allo**graft; that is, cells harvested from another person, usually a family member sharing the same major histocompatibility molecules.

Allografted hematopoietic stem cells also sometimes fail to cure, but in that case it is because not all of the patient's leukemic cells were destroyed. However, an infusion of T lymphocytes from the blood of the same donor that provided the cells can finish off the job.

This effect is called the **graft-versus-leukemia** effect.

However, most (if not all) of the donor T cells are probably attacking normal cell surface molecules, not tumor-specific ones. (Even if the donor and recipient are matched for the major histocompatibility molecules, there will be minor ones that elicit a rejection response.)

So the patient may also suffer life-threatening graft-versus-host disease (GVHD).

The graft-versus-leukemia effect lays the foundation for an approach that has shown considerable promise against various blood cancers and even some solid (e.g., kidney) tumors.

- The patient is treated to kill some — but not all — of the bone marrow cells (nonmyeloablative conditioning).
- Instead of using high doses of radiation to the entire body and chemotherapy, only the lymphoid organs (spleen, thymus, lymph nodes) are irradiated (called "total lymphoid irradiation").
- Antithymocyte globulin can also be given.
- Even though this leaves some cancer cells, it makes it possible for allogeneic bone marrow stem cells to take up long-term residence in the recipient (just as immunosuppression allows kidney transplants, etc. to avoid rejection by the recipient).
- This is followed by an infusion of T cells from the same donor. These can then go to work against the cancer cells without being threatened with rejection by the host.
- Once again, though, they will also attack normal cells of the recipient usually causing graft-versus-host disease (GVHD). However, this promises to be milder than that following myeloablative conditioning — perhaps because repeated small doses of radiation favors the survival of natural killer (NK) cells, and these appear to protect against GVHD.

In mice, the graft-versus-leukemia effect can be enjoyed without the downside of GVHD by including extra-large numbers of regulatory T cells (Treg cells) in the bone marrow infusion. Whether this approach could be helpful for humans remains to be seen.

Virotherapy

It has long been known that viral infections can occasionally (and unpredictably) cause tumors to regress. A number of viruses have been studied in the hope of developing a reliable therapy. On 27 October 2015, the U.S. FDA approved T-VEC (Imlygic®) for the treatment of melanoma. T-VEC is a mutated and engineered Herpes Simplex Virus (HSV-1 — the cause of cold sores). The alterations in the virus include incorporating the gene for GM-CSF and a mutation that prevents the virus from infecting non-dividing cells while preserving its ability to infect and replicate in cancer cells. Replication kills the cells and causes them to release:

- more viruses which spread the infection;
- tumor antigens, and
- GM-CSF which attracts dendritic cells to the site. These take up the tumor antigens and present them to T cells that go on to mount an attack against surviving tumor cells.

(Tumor cell death by HSV does not qualify it as immunotherapy, but the T-cell response that results certainly does.)

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12.10: Cancer- The Causes and Prevention of Cancer

As described by Bruce N. Ames, Professor of Biochemistry & Molecular Biology, Director of the National Institute of Environmental Health Sciences Centerm, University of California at Berkeley

Aging is in good part due to the oxidants produced as by-products of normal metabolism. These oxidants, such as superoxide and hydrogen peroxide, are the same mutagens produced by radiation, and cause damage to DNA, proteins, and lipids.

The DNA in each cell of a normal rat receives on average about 100,000 oxidative lesions per day. DNA repair enzymes constantly remove this damage, but they do not keep up: a young rat has about one million oxidative lesions in the DNA of each cell, which increases to about two million in an old rat. A human cell receives about ten times less damage than a rat cell, in agreement with the higher cancer rate and shorter lifespan of a rat.

The degenerative diseases of aging such as **cancer**, cardiovascular disease, cataracts, and brain dysfunction, are increasingly found to have, in good part, an oxidative origin. It is argued that dietary antioxidants, such as Vitamins C and E and carotenoids, play a major role in minimizing this damage and that most of the world's population is receiving inadequate amounts of them, at a great cost to health.

The main source of dietary antioxidants is fruits and vegetables. Humans should eat 5 portions of fruits and vegetables per day, yet only 9% of the U.S. population eats that much. Epidemiological studies show that the incidence of most types of cancer is double among people who eat few fruits and vegetables as compared to those who eat about five portions per day. Considerable evidence indicates that oxidative damage is important in cardiovascular disease, cataracts, and brain and immune system dysfunction, and that adequate dietary antioxidants can minimize their incidence.

Men with low Vitamin C intake have low vitamin C in their seminal fluid and much more oxidative damage to the DNA in their sperm. Male smokers are particularly at risk as they have depleted antioxidant pools (cigarette smoke is extremely high in oxidants). A smoker must eat two to three times as much Vitamin C as a non-smoker to maintain an equal plasma level, yet smokers tend to eat worse diets than non-smokers. Indeed, male smokers have a considerably higher risk of having children with birth defects and childhood cancer.

The **three main causes of cancer** are **smoking**, dietary imbalances (**excess fat and calories**; inadequate intake of fruits, vegetables, fiber, and calcium), and chronic infections leading to **chronic inflammation** (hepatitis B and C viruses, Helicobacter pylori infection, schistosomiasis, etc.). Chronic inflammation is a major cause of cancer in the world because it releases powerful oxidants which both **stimulate cell division** and are **mutagens**.

Past **occupational exposures** might cause about 2% of current human cancer, a major part being asbestos exposure in smokers, and industrial or **synthetic chemical pollution** causes less than 0.1 %, in my view. The age-adjusted cancer death rate in the U.S. for all cancers combined (excluding those attributable to smoking) has been remaining steady since 1950, while life expectancy increases every year.

We are the healthiest we have ever been in human history.

Two factors are critical in the formation of mutations: lesions in DNA, formed when DNA is damaged, and **cell division**, which converts DNA lesions to **mutations**. Agents increasing either lesions or cell division increase mutations and as a consequence increase cancer incidence. **Hormones stimulating cell division** increase cancer incidence (e.g., levels of estrogen in breast cancer and testosterone in prostate cancer); hormones may be a risk factor in about 20% of human cancer.

Animal cancer tests, which are done at the maximum tolerated dose (MTD), are being misinterpreted to mean that low doses of the chemicals tested and found positive are thereby relevant to human cancer. Animal cancer tests are mainly done on synthetic chemicals and industrial pollutants, yet **half of all natural chemicals** that have been tested at the MTD are rodent carcinogens.

It is argued that the explanation for the high frequency of positive results in animal cancer tests is that high dose animal cancer tests are mainly **measuring increases in cell division** due to cell killing and compensatory cell division; this is a high dose effect that **does not occur at low doses**.

In any case 99.9% or more of the chemicals we eat are natural. For example, **99.99% of the pesticides we eat are natural chemicals that are present in plants to ward off insects and other predators**. More than half of those natural pesticides

tested in high dose animal tests are rodent carcinogens. There are about 10,000 or so different natural pesticides in our diet, and they are usually present at enormously higher levels than synthetic pesticides.

Cooking food also generates thousands of chemicals. There are over 1000 chemicals reported in a cup of coffee. Only 26 have been tested in animal cancer tests and more than half are rodent carcinogens; there are still a thousand chemicals left to test. The amount of potentially carcinogenic **pesticide residues** consumed in a year is less than the amount known of rodent carcinogens in a cup of coffee.

The reason we can eat the tremendous variety of natural chemical rodent carcinogens in our food is that animals are extremely well defended against all chemicals by many general defense systems. These enzymes, e.g., DNA repair and glutathione transferases which defend against reactive compounds such as mutagens, are all inducible (more of them are made when they are in use). They are **equally effective against natural and synthetic** reactive chemicals. Thus, animals are extremely well defended against low doses of chemicals. One does not expect, nor does one find, a general difference between synthetic and natural chemicals in their carcinogenicity, and though less well studied, the same would be expected for mutagenicity, teratogenicity, and acute toxicity.

The effort to eliminate synthetic pesticides because of unsubstantiated fears about residues in food will make fruits and vegetables more expensive, decrease consumption, and thus increase cancer rates. The levels of synthetic pesticide residues are trivial in comparison to natural chemicals, and thus their potential for cancer causation is extremely low.

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12.11: Estimating Cancer Risks

Is there a safe dose of any mutagen or carcinogen?

We live surrounded by radiation and by chemicals that cause mutations in test organisms (like bacteria, yeast, and mice) and cause an increase in the rate of cancers in experimental animals (rats and mice). Is there any safe dose for humans of these agents (which include oxygen!) The question is exceedingly difficult to answer and, I believe, at low doses, unanswerable. Why?

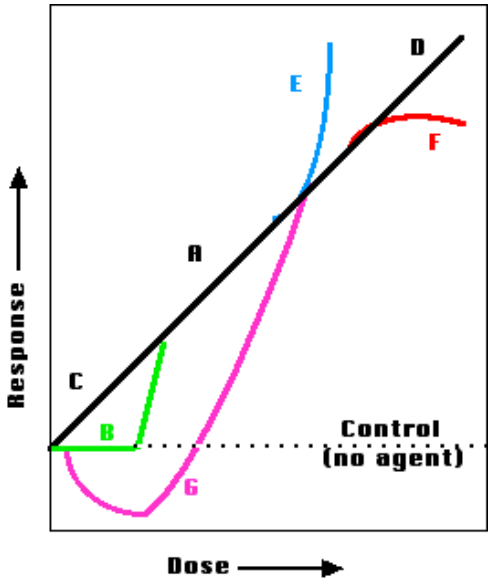


Figure 12.11.1: Dose - Response relationship

Figure 12.11.1 shows several theoretical dose-response relationships. There is considerable evidence that at **moderate doses** of a mutagen or carcinogen, the response is linear (**A**). However, at very **low** doses of some chemicals, there may be a threshold below which the agent has no effect (**B**). Many workers believe that for some agents, it is likely that even the tiniest doses will have an effect (**C**), but the population exposed must be large enough to observe it. This is called the linear no-threshold (**LNT**) model. Note that even at zero dose, the line does not intercept the origin. This is because even unexposed animals (including people) show a spontaneous level of response (e.g., tumors).

There is also evidence that for some agents in some circumstances, increasing the dose (at relatively low levels) actually reduces the response below control levels (**G**). This phenomenon is called **hormesis**. At very high doses, the rate of response may increase faster than the dose (**E**) as, for example, the probability of a single cell suffering **two** mutations increases. On the other hand, very high doses may kill off damaged cells before they can develop into tumors (**F**).

Radiation and cancer

High doses of radiation cause cancer. Various studies, including excellent ones on the survivors of Hiroshima and Nagasaki, show that a **population** exposed to a dose of 100 millisieverts (mSv) will have a measurable increase (about 1%) in the incidence of cancer. Note that the measurements are made on a population, not on individuals. We can never say that a particular individual exposed to a particular dose of radiation will develop cancer. The induction of cancer is a chance ("stochastic") event unlike the induction of radiation sickness which is completely predictable. The element of chance arises because cancer is an event that occurs in a single cell unlucky enough to suffer damage to several specific genes. However, the energy needed to cause mutations is very low. So if you expose a sufficiently large number of cells to even tiny doses of radiation, some cell is going to be unlucky. How can we evaluate the risk?

Collective Dose

100 mSv causes a 1% increase in cancer in a population; i.e., it should cause an increase of 1 cancer in every 100 people in the exposed population. But if our reasoning is correct, a population of 10,000 people exposed to 1 mSv should also yield one case of radiation-induced cancer. In **any** population, where the product of radiation dose (in mSv) times population size equals 1 x 10^4, one case of cancer will be induced. The product of exposure multiplied by the size of the exposed population is known as the **collective dose**. Its units are (persons)x(mSv).

Example 12.11.1

The population of the U.S. in 2009 was about 305 million. so anything that increases the annual exposure of the U.S. population by as little as 0.01 mSv (a typical chest x ray is 0.02 mSv) per year would cause an additional 305 cases of cancer.

$$\frac{(305 \times 10^6\, persons)(0.01\, mSv)}{1 \times 10^4\, person\, mSv/cancer} = 305\, cancers \tag{12.11.1}$$

But consider:

- The total number of cancer deaths in the United States that year was expected to exceed 560,000.
- How can we possible detect an increase of 305 faced with these large numbers?

Hormesis?

The citizens of Colorado are exposed to background radiation of some 1.8 mSv per year; the figure for Massachusetts is only 1.02 mSv/year.

- If the linear non-threshold model is correct, we would expect to find a higher incidence of cancer in Colorado than in Massachusetts.
- If the background radiation added to other sources keeps both groups below a threshold, then we would expect no difference in cancer incidence.
- If the modest increase in background radiation in Colorado has a protective effect (hormesis), then we would expect that their cancer incidence would be lower than in Massachusetts.

What do we find? In 1999, when adjusted for the age of the population, the incidence of cancer averaged 16% higher in Massachusetts than in Colorado. (If this truly **is** evidence of hormesis, the mechanism is unknown.)

Some other parts of the world have background radiation levels that dwarf those in Colorado. In some houses in Ramsar, Iran, the inhabitants are exposed to an annual dose of background radiation of as much as 130 mSv per year — over 70 times that in Colorado. Nevertheless, the inhabitants of Ramsar are just as healthy as — or even healthier than — control populations exposed to far lower levels of radiation.

Chernobyl

It has been estimated (in this case, using a collective dose value of 5 x 10^4 person mSv/cancer) that the radioactive fallout from the nuclear accident at Chernobyl (now often spelled "Chornobyl") in 1986 will cause an increase of 17,000 cancers over the lifetime of people living in the Northern Hemisphere.

Large those this estimate seems, it is dwarfed by the 513 million cancer deaths that will occur anyway in this population. Even among those heavily exposed (rescue workers and people living in the region), the expected death toll from cancer is ~4,000 or only 3% more than their death rate from cancer would have been anyway. This is why I say above that the answer to the question of the dangers of low doses of radiation is unknowable.

> As of September 2005, some 4000 children and adolescents who drank milk contaminated by the radioactive iodine [^{131}I] released in the accident had come down with thyroid cancer. In their case, the ability of the thyroid gland to concentrate iodine within its cells resulted in those cells receiving a relatively high, not a low, dose. As of that date, however, only 15 of those cancer patients had died.

Chemicals and cancer: dioxin

At one time it was found that the chemical dioxin, which can be produced as a contaminant in the manufacture of paper and cardboard, was leaching from milk cartons into milk itself.

- the concentration in the milk averaged 0.1 part per trillion (ppt) or 0.0001 µg in a liter (10^9 µg) of milk. Assuming:
  - 0.1 µg per day given to rats increases their rate of tumors by 1%
  - the idea of **collective dose** applies to chemicals (that is, a single molecule in an unlucky cell can turn it cancerous)
  - people are 100 times more sensitive to dioxin than rats (probably not true) and
  - people are 100 times larger than rats
- we conclude that there is a risk of 10 additional cancers in every million people consuming a liter (about a quart) of milk a day from cardboard containers.

And, in fact, this was the estimate made. The uncertainties in such assumptions helps explain the controversy that has so often swirled around the test data on such chemicals as

- the artificial sweeteners cyclamate and saccharin,
- the pesticide Alar,
- the hydrocarbons in a charcoal-broiled steak,
- the chlorinated compounds in municipal water supplies.

Some chemicals appear to have a safety threshold

Cells have a number of different methods for detoxifying certain types of chemicals. So long as these mechanisms are not overwhelmed, they should provide a threshold of safety.

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12.12: The LD50 test

The LD50 is a standardized measure for expressing and comparing the toxicity of chemicals. The LD50 is the dose that kills half (50%) of the animals tested (LD = "lethal dose"). The animals are usually rats or mice, although rabbits, guinea pigs, hamsters, and so on are sometimes used. In all these tests, the dose must be calculated relative to the size of the animal. The most common units are milligrams of chemical per kilogram of test animal (mg/kg or ppm).

Table 12.12.1*: LD_{50} values of common drugs*

Chemical	Category	Oral LD_{50} in Rats (mg/kg)
Aldicarb ("Temik")	Carbamate	1
Carbaryl ("Sevin")	Carbamate	307
DDT	Chlorinated hydrocarbon	87
Dieldrin	Chlorinated hydrocarbon	40
Diflubenzuron ("Dimilin")	Chitin inhibitor	10,000
Malathion	Organophosphate	885
Methoprene	JH mimic	34,600
Methoxychlor	Chlorinated hydrocarbon	5,000
Parathion	Organophosphate	3
Piperonyl butoxide	Synergist	7,500
Pyrethrins	Plant extract	200
Rotenone	Plant extract	60

Table 12.12.1 gives the LD50 values for some insecticides. In each case, the chemical was fed to laboratory rats. Note that the lower the LD50, the more toxic the chemical. Even adjusting for the test animal's weight, the LD50 for one species is often quite different from that for another. Thus any LD50 value gives only a rough estimate of the risk to humans. The way in which the chemical is administered also has a marked effect on LD50 values. The chemical may be fed, injected, applied to the animal's skin, etc., and each method usually generates a different LD50.

> Because a single test may kill as many as 100 animals, the United States and other members of the Organization for Economic Cooperation and Development agreed in December 2000 to phase out the LD50 test in favor of alternatives that greatly reduce (or even eliminate) deaths of the test animals.

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12.13: Dioxin

Name given members of a family of closely-related chemicals. The term dioxin is often used for one of these: 2,3,7,8-tetrachlorodibenzo-p-dioxin or TCDD. This substance was present as a contaminant in the herbicide **agent orange**, which was so widely used during the Vietnam war.

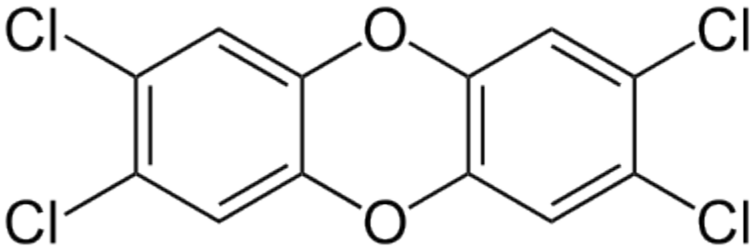


Figure 12.13.1: Structure of 2,3,7,8-tetrachlorodibenzo-p-dioxin.

When ingested or injected, TCDD is extremely poisonous to laboratory animals. At sub-lethal concentrations, it causes cancer and birth defects in them. Exposure to high levels of dioxins causes a severe skin disease (chloracne) in humans as well as damage to the liver and nervous system. While the evidence is still hotly debated, the U.S. Environmental Protection Agency (EPA) is convinced that dioxins cause cancer in humans. They base this conclusion on extrapolating from dose-response studies done in animals (rats) and following the health of industrial workers who were exposed to dioxins in the U.S., Germany, and the Netherlands.

Thanks to the development of delicate analytical techniques, it is possible to detect trace amounts in everyone's blood. Most of us have a few parts per trillion (ppt) of TCDD in our serum. TCDD (and other dioxins) are produced when organic matter is burned. Measurable levels are found in soot from wood-burning stoves and the ash of municipal incinerators. However, the amounts to which we are exposed have dropped some threefold since the mid-80s, and the cancer risk dioxins pose for most of us is probably close to zero.

Dioxin can prevent disease! (in mice). Experimental allergic encephalomyelitis (**EAE**) is a disease in experimental animals (e.g., mice, guinea pigs) that closely mimics multiple sclerosis, an autoimmune disease of humans in which the myelin sheaths of neurons are destroyed. In the 1 May 2008 issue of **Nature**, F. J. Quintana and colleagues reported that they could strongly suppress the induction of EAE in mice by pretreating them with 1 µg of TCDD. The protection appeared to be mediated by regulatory T cells (Treg) whose numbers rose sharply following TCDD treatment.

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12.14: Magnetic Fields and Cancer

The Background

Late in the 1970s, researchers investigating a cluster of cancers in children in Colorado found an association with living near high-voltage power lines. The cancers, a form of leukemia called **acute lymphoblastic leukemia** (**ALL**), had long been associated with exposure to ionizing radiation. But power lines do not generate ionizing radiation. What they do generate is a weak magnetic field that oscillates at the frequency of the alternating current (60 hertz in the U.S.; 50 hertz in Europe). Follow-up studies elsewhere continued to find a weak association between living near power lines and the incidence of ALL in children. Note that the association was with the proximity to power lines; not to the strength of the magnetic fields. The nature of the wiring (e.g., voltage, proximity) was used as a **surrogate** to the actual agent under suspicion (the magnetic field).

The National Cancer Institute (NCI) Study

On July 3, 1997, The New England Journal of Medicine published the largest and best study of the question (Martha S. Linet, et al, "Residential Exposure to Magnetic Fields and Acute Lymphoblastic Leukemia in Children").

Their conclusion: "Our results provide little support for the hypothesis that living in homes with high time-weighted average magnetic fields or in homes close to electrical transmission or distribution lines is related to the risk of childhood ALL."

How the NCI study differed from earlier studies

The NCI study differed from the earlier studies in 4 important ways:

- It involved a much larger sample size (624 children with ALL and 615 children chosen at random to compare their homes with those of the patients.
- The strength of the magnetic fields in the homes were actually measured (including continuous measurement for 24 hours under the child's bed). They also evaluated the nearby power lines as the earlier studies had done.
- The collection of data was "blinded"; that is, the people doing the measurements did not know whether they were in the house of an ALL patient or in the house of a control.
- The investigators had no axe to grind. None had any connection to the power industry or to grieving parents seeking to find an explanation for the tragedy that had struck their family.

The Magnetic Field Results

Patients and controls were grouped in 7 classes ranging from a magnetic field of less than 0.065 microteslas (μT) to greater than 0.5 μT. The tesla is a unit of magnetic field strength; the earth's magnetic field, which makes a compass needle turn, is about 50 microteslas (but does not fluctuate at 60 hertz as the much smaller fields near alternating current lines do). The Odds Ratio is a calculation of how likely it is that the results for the patient group differ from that of the control group. The total number of patients and controls in each class is shown within each bar. The blue lines show the 95% confidence limits; that is, that there is a 95% probability that the "true" mean (the height of the bar) is somewhere within the range shown in blue.

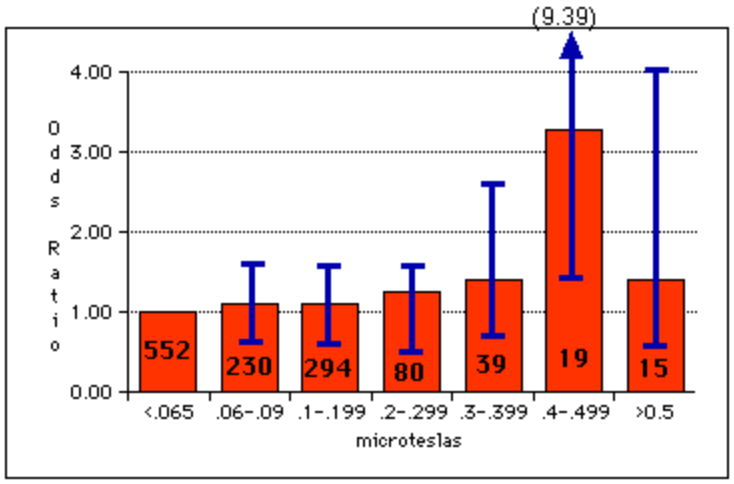


Figure 12.14.1: Odds Ratio

Interpreting the results

Only one class of exposure (0.400 - 0.499 µT) showed a statistically significant difference between patients and controls. Is it truly significant? Perhaps. But note that only 19 children of the 1,239 enrolled in the study lived in homes with this level of magnetic field.

How do this and the earlier studies meet the 5 standards of epidemiology?

1. **High Relative Risk**
   Not met. Every group but one had a relative risk whose 95% confidence limit included 1.00; that is, no relative risk at all.
2. **Consistency**
   Not met.
   - The earlier studies did not measure magnetic fields.
   - Most of the earlier studies, which simply evaluated the nature of the nearby power lines, showed a 2–3 fold increase in relative risk whereas this study showed no increase.
3. **A graded response to a graded dose**
   Not met. There is no steady increase in ALL with increasing exposure to magnetic fields. The possible increased risk of ALL with exposure to 0.400 - 0.499 µT is followed by no increase at exposures above 0.5 µT.
4. **Temporal relationship**
   Met. In fact, built into the design of the study. All the patients were selected after they had developed ALL.
5. **A plausible mechanism Not met**. In vitro studies have failed to reveal any mechanism to explain how such weak magnetic fields could produce oncogenic changes in cells. (Note that the Y axis of this graph of representative magnetic fields is logarithmic: the magnetic field directly under a high-voltage power line is only 1/10 that of the earth's own magnetic field.)

   Two papers published in 1992 claimed that weak magnetic fields increase the flow of calcium ions into lymphocytes. Such a response may trigger mitosis and thus provide a plausible mechanism for a tumor-promoting effect. However, in June 1999 the author was censured by the Office of Research Integrity for falsifying his data, and the author retracted the papers.

The Bottom Line

In the words of Edward W. Campion, M.D. (New England Journal of Medicine, 337:44, July 3, 1997):

"there is no convincing evidence that high-voltage power lines are a health hazard or a cause of cancer...18 years of research have produced considerable paranoia, but little insight and no prevention. It is time to stop wasting our research resources. We should redirect them to research that will be able to discover the true biologic causes of the leukemic clones that threaten the lives of children."

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UNIT 13: AGING

13.1: Aging
13.2: Telomeres

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13.1: Aging

What is Aging?

Aging is the progressive loss of physiological functions that increases the probability of death. This table gives some data.

Loss of structure and function in aging. Figures represent percentage of a given function remaining in an average 75-year-old man compared with that found in an average 30-year-old man, the latter value taken as 100%.

Weight of brain	56%
Blood supply to brain	80
Output of heart at rest	70
Number of glomeruli in kidney	56
Glomerular filtration rate	69
Speed of return to normal pH of blood after displacement	17
Number of taste buds	36
Vital capacity	56
Strength of hand grip	55
Maximum O_2 uptake during exercise	40
Number of axons in spinal nerve	63
Velocity of nerve impulse	90
Body weight	88

The decline in function certainly occurs within cells. This is especially true of cells that are no longer in the cell cycle:

- neurons in the brain;
- skeletal and cardiac muscle;
- kidney cells.

Tissue and organs made of cells that are replenished by mitosis throughout life. Blood and intestinal epithelium show far fewer signs of aging.

In the natural world, very few animals live long enough to show signs of aging. Random mortality from starvation, predation, infectious disease and a harsh environment (e.g., cold) kills off most animals long before they begin to show signs of aging. Even for humans, aging has only become common in recent decades.

At the start of the 20th century, infectious diseases such as pneumonia and influenza caused more deaths in the United States than "organic" diseases like cancer. Now the situation is reversed. The availability of effective weapons against infectious disease (e.g., sanitation, antibiotics, and immunization) has greatly increased the average life span (but not maximum life span) and resulted in "organic" diseases like cardiovascular disease and cancer becoming the most common cause of death.

In 1900, a newborn child in the U.S. could look forward to an average life expectancy of only 47 years. Infectious diseases were the major causes of death, killing most people before they reached an age when aging set in. Three-quarters of a century later, life expectancy had risen to 73 years and "organic" diseases, including all the diseases of aging, had replaced infectious diseases as the major cause of death. Today, the life expectancy has risen to 80 for women (74 for men), and coping with an aging population has become a major economic and social challenge in the U.S.

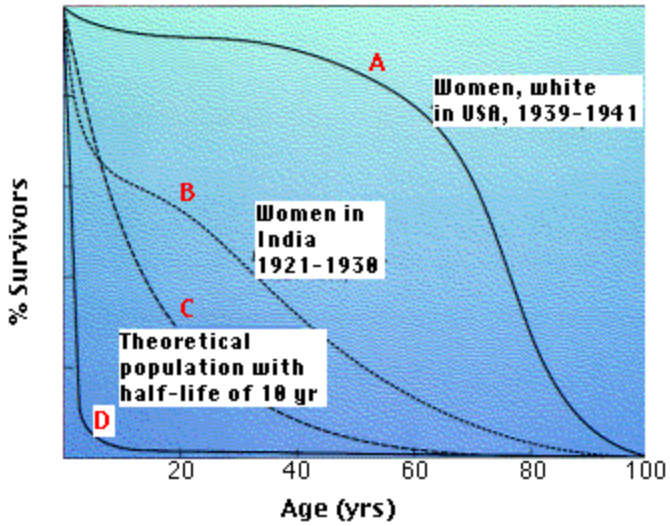


Figure 13.1.1 Survival curves

The above graph shows four representative survival curves. The vertical axis represents the fraction of survivors at each age (on the horizontal axis).

- Curve **A** is characteristic of organisms that have low mortality until late in life. Then mortality increasingly becomes the endpoint of the aging process.
- Curve **B** is typical of populations in which such environmental factors as starvation and disease obscure the effects of aging (and infant mortality in high).
- Curve **C** is a theoretical curve for organisms for which the chance of death is equal at all ages. This might be the case for organisms that show few, if any, signs of aging (some fishes) or those (e.g., songbirds in the wild) that suffer severe random mortality from environmental causes throughout life.
- Curve **D** is typical of organisms, oysters for example, that produce huge numbers of offspring accompanied by high rates of infant mortality.

Organisms with survivorship curves between **C** and **D** have no opportunity to show the signs of aging.

Aging in Invertebrates

Invertebrate animals have provided some important clues about the aging process.

- Colonial invertebrates like sponges and corals don't show signs of aging. Even individual cnidarians, like the sea anemone that lived for 78 years, show little or no sign of aging. In all these cases, this is probably because there is constant replacement of old cells by new ones as the years go by.
- Lobsters also can live to a very old age with no obvious sign of a decline in fecundity or any other physiological process. But lobsters never stop growing, so once again it may be the continuous formation of new cells that keeps the animal going.
- In culture vessels, **Drosophila** does have a limited life span and shows signs of aging before it dies. Two factors have been found to influence the aging process and thus life span:
  - **Calorie restriction**, that is, a semi-starvation diet. In fact, restricting food intake has been shown to increase life span (and slow aging) in all animals — including mammals — that have been tested.
  - Single genes have been identified that extend life span in Drosophila (and also in the invertebrate *Caenorhabditis elegans*).

Aging in Vertebrates

Some cold-blooded vertebrates fishes, amphibians, reptiles have long life spans if they can survive environmental hazards (giant tortoises are known to have reached 177 years of age). These animals are cold-blooded and grow so slowly that they will probably succumb to environmental hazards before they stop growing and begin to show signs of aging. The situation is different for **birds** and **mammals**. They are warm-blooded, grow rapidly to adult size and, if protected from environmental hazards, will show signs of aging.

Why Do We Age?

Programmed in our genes

The pros

- Single genes have been found that increase life span in Drosophila, *C. elegans*, and mice. Genes that suppress signaling by insulin and insulin-like growth factor-1 (Igf-1) increase life span in these animals. Examples:
  - Mice with one of their Igf-1 **receptor** genes "knocked out" live 25% longer than normal mice.
  - **Klotho**. Cells in the kidney and brain release the extracellular portion of a cell surface transmembrane protein into the blood. This "hormone", called Klotho, binds to receptors on many target cells reducing their ability to respond to insulin and Igf-1 signaling.
    - Mice homozygous for a **mutant** *klotho* gene show many signs of premature aging while
    - mice expressing extra-high levels of the Klotho protein live 20–30% longer than normal.
- Long life spans clearly run in human families.
- Aging often appears sooner in animals that suffer high death rates from external causes (e.g. predation) early in life.

  Why should this be? Three (interrelated) possibilities:

  - The accumulation of harmful mutations (in the germline). Few individuals survive long enough for these to be selected **against**.
  - Antagonistic pleiotropy. Genes that promote survival early in life at the expense of maintaining the body will be selected **for**.
    Some examples:
    - ***p53***. By forcing cells with damaged DNA to stop dividing and become senescent or even to die by apoptosis, it protects the organism from the threat of those cells becoming cancerous but at the expense of reducing cell renewal (e.g., by decreasing the size of the pools of stem cells). Mice that are forced to produce higher-than-normal levels of the p53 protein show many signs of premature aging while female mice that are deficient in p53 have reduced fecundity (blastocysts fail to implant). So here is a tradeoff by a gene that promotes evolutionary success at an early age but at the expense of accelerated aging.
    - In both Drosophila and *C. elegans*, some mutations that increase life span do so at the cost of decreased fecundity and vice versa.
  - Disposable soma. Early death from external causes will select for genes that increase the chances of passing germplasm on (i.e. reproduction) at the expense of genes that might delay aging.
- There is no way that natural selection can select for genes whose only beneficial effect appears after the age of reproduction is over. But
  - any genes that extend the reproductive period or
  - any genes that promote fitness in youth as well as longevity

  would be selected for.

The cons

High early mortality from external causes (e.g. predators) has been linked to early aging (in the survivors) in some animals, but the reverse has been found in others. These contradictory results do not negate the role of genes in aging, but indicate that other environmental factors (e.g. more food left for the survivors) may skew the outcome.

The Inevitable Consequence of an Active Life

The pros

- Many cold-blooded vertebrates (e.g., many fishes and reptiles) do not show signs of aging.
- Transgenic mice whose "thermostat" in the hypothalamus has been reset to give a lower body temperature (reduced by 0.3 – 0.5°C) live 12% (males) to 20% (females) longer than their nontransgenic littermates. This translates into adding some 3 months to the average life span of 27 months for these mice. (See Conti, B., *et al.*, **Science**, 3 November 2006).)
- The effects of **Calorie Restriction** (CR). The life span of yeast, *C. elegans*, Drosophila, birds, and mammals (mice, rats, and

probably monkeys) can be extended, and signs of aging delayed, if they are maintained on a semi-starvation diet. Calorie restriction in mice causes

- a drop in
  - the level of circulating insulin and insulin-like growth factor-1 (Igf-1);
  - the level of **glucose** and triglycerides in the blood;
  - the level of NADH (produced by cellular respiration) within cells;
- an increase in the production of **sirtuins** — deacetylases that remove acetyl groups from proteins.
- apoptosis of cells to be inhibited;
- formation of adipose tissue to be suppressed;
- increased production of nitric oxide (NO) which is essential for the benefits of CR to take effect.
- greatly increased physical activity and
- lower body weight.

The life-extending and other effects of CR on rats and mice may not be as significant as they seem. All the studies have been done on laboratory rats and mice. Control animals are normally fed *ad libitum*, which means that they have food available all the time. But this would not likely be the case in the wild so it may be that the physiology and life span of these animals are already compromised and that the effects of CR are mainly restoring normal conditions for these species.

The problem of proper controls may also explain the divergent results in studies of CR in rhesus monkeys.

- In the 10 July 2009 issue of **Science**, researchers at the Wisconsin National Primate Research Center reported the status of rhesus monkeys that had been on CR for 20 years compared with a control group allowed to feed *ad libitum* on the same diet during that time. The results: the CR animals showed markedly fewer signs of aging (none showed any signs of diabetes), and the few that died from age-related causes did so at only one-third the rate of the controls.
- However, a 25-year study of CR in rhesus monkeys carried out at the National Institute of Aging (NIA) (and reported in **Nature** in August 2012) showed no increased longevity in the CR monkeys.

The difference may have arisen because the control monkeys in Wisconsin were fed *ad libitum* while the controls at the NIA were fed a fixed amount. Furthermore, the diet used in the Wisconsin study were much higher in sugar than the diet at the NIA, and the NIA diets also included omega-3 fatty acids and other healthy components absent from the Wisconsin diet. So perhaps the positive effect seen in the Wisconsin study resulted from the CR animals simply getting less of an unhealthy diet than the controls.

Resveratrol

Resveratrol, is a small molecule found in red wine which appears to activate sirtuins mimicking the effects of calorie restriction. Mice given daily doses of resveratrol while indulging in a high-fat diet get fat but avoid the degenerative changes and shortened life span that normally accompany a high-fat diet. But before rushing out to buy red wine, realize that the doses of resveratrol given to the mice were far higher than could be supplied by drinking it. Studies on the effect of resveratrol on extending life span in yeast, Drosophila and *C. elegans* have produced mixed results.

No one knows for certain why calorie restriction delay aging, but some mechanisms might be because it lowers the level of glucose in the blood and thus the speed with which lipids and proteins suffer from glycation. **A**dvanced **g**lycation **e**nd products (AGEs) are molecules that have reduced function because of the haphazard addition of sugars to them. For proteins like collagens and elastin, this results is increasing stiffness of the extracellular matrix (ECM) of blood vessels, joints, heart, kidney, etc. Reducing calorie intake reduces female fecundity (at least in *C. elegans*, Drosophila, rats and mice). The energy that would have been devoted to producing offspring can be devoted instead to tissue repair and maintenance. Calorie restriction raises the level of **sirtuins:**

- The SIRT1 protein CR in knockout mice lacking SIRT1 gain no increase in life span.
  - plays a key role in repairing DNA damage and so helps protect the integrity of the genome which appears to be essential to longevity.
  - It also inhibits the nutrient sensor TOR ("target of rapamycin") which accelerates aging in mice.
  - SIRT1 also inhibits p53 activation thus protecting against mitochondrial damage.
- The SIRT3 protein is found in mitochondria where it inhibits the production of **free radicals**.

The Free Radical Theory of Aging

A major aspect of metabolism is the oxidation of foodstuffs by the mitochondria. Electron transport in the mitochondria generates **reactive oxygen species** ("**ROS**") such as the superoxide anion ($O2^-$), which generates hydrogen peroxide ($H2O2$). Although cells contain enzymes for detoxifying these reactive substances (e.g., catalase which breaks down $H2O2$), they eventually and inevitably damage macromolecules in the cell: proteins; lipids; and probably most important of all, **DNA**.

Damaged proteins and lipids accumulate in the cell, especially nondividing cells like neurons and muscle, producing aggregates of denatured proteins and an "aging pigment" called **lipofuscin** (a principal component of ear wax). The accumulation of protein aggregates in striated muscles reduces muscle strength. Protein aggregates accumulate more slowly in the cells of animals on a calorie restricted diet — perhaps as a result of more-efficient autophagy. However, it may be damage to DNA that is the crucial factor in the decline in cell function with age.

The DNA of the mitochondria (mtDNA) may be at special risk. ROS are produced as an inevitable byproduct of electron transport in the mitochondria and thus are generated close to the mtDNA. But the products of these genes are essential for electron transport. So perhaps a positive feedback loop is generated: ROS -> mutations in electron transport genes reducing their efficiency -> more ROS production.

Supporting evidence:

- mtDNA accumulates mutations faster than nuclear DNA, and these show the chemical characteristics of damage by ROS.
- Transgenic mice containing the human gene for catalase (but with the targeting signal that would normally send the protein to peroxisomes replaced with that for mitochondria) live 20% longer than normal for their strain. [See Schriner, S. E. *et al.*, **Science**, 24 June 2005.]
- Transgenic mice whose DNA polymerase for copying mtDNA genes (DNA polymerase gamma) is defective, and introduces an elevated number of mutations in mtDNA, show many signs of premature aging — both cellular and in various organ systems — and die early.

The cons

- Neither mice genetically engineered to overproduce free radicals, nor those engineered to produce lower amounts of free radicals, have any change in their lifespan.
- Bats and mice are similar in size and metabolic rate, but bats can live ten times as long.
- Although glucose-starved yeast do live longer, they have an increased — not decreased — rate of cellular respiration.
- The metabolic rate of mice on a CR diet is no lower than that of mice on a normal diet.
- The beneficial effects of CR take hold at any time, at least in Drosophila. Even after three weeks on a rich diet (in the second half of the normal life span of adult flies), switching to a CR diet reduces mortality to the same degree as flies maintained on CR throughout their adult lives. The reverse is also true — switching from a CR diet to a rich diet quickly undoes the good work of the former. These results suggest that if a rich diet does produce irreversible and accumulating damage, its harmful effects on life span can be blunted at any time.

The Accumulation of Senescent Cells

Chronological Senescence

Once formed, some cells in a mouse or human are never replaced. A neuron formed during embryonic development may still be functioning at the end of life. However, during its life span, damage to its organelles and DNA may accumulate resulting in a loss of function. This is called chronological senescence. In other tissues, e.g., blood and epithelia, new cells replace old ones throughout life. But even though new, they may have reduced function because of replicative senescence.

Replicative Senescence

One might expect that cells removed from a mouse or human and placed in tissue culture could be cultured indefinitely, but that is not the case. When human fibroblasts, for example, are placed in culture, they proliferate at first, but eventually a time comes when their rate of mitosis slows and finally stops. The cells continue to live for a while, but cannot pass from G1 to the S phase

of the cell cycle. This phenomenon is called **replicative senescence**. Fibroblasts taken from a young human pass through some 60–80 doublings before they reach replicative senescence.

Why should this be? Cells — unless they retain the enzyme telomerase — lose DNA from the tips of their chromosomes (telomeres) with each cell division. In general, the telomeres in the cells of old animals are much shorter than those in young animals. A recent study of short-lived versus long-lived birds showed that telomere shortening was faster in the short-lived species. And one species, a petrel which lives four times as long as other birds of its size, actually has telomeres that grew longer with age. Most somatic cells of the body cease to express telomerase. However, cells genetically manipulated to express telomerase long after they should have stopped, avoid replicative senescence. Germline cells, e.g., spermatogonia, and some stem cells continue to express the enzyme. Some 95% of cancer cells express telomerase. If telomeres get too short (less than 13 repeats in human cells), chromosome abnormalities — a hallmark of cancer — occur. Cancer can be avoided if the cell senses this dangerous condition and ceases to divide. So telomere shortening may protect against cancer at the price of cell senescence.

Two proteins encoded by tumor suppressor genes p53 and $p16^{INK4a}$ play pivotal roles in stopping the cell cycle. The result: replicative senescence. So replicative senescence may be the price we pay for removing cells from the cell cycle before they can accumulate the mutations that would turn them into cancer cells.

The role of the tumor suppressor proteins in replicative senescence is mirrored in the intact animal, at least in mice.

- Mice engineered to express abnormally-high levels of p53 activity show many signs of premature aging including premature reduction in the length of their telomeres.
- Mice
  - that express abnormally-high levels of $p16^{INK4a}$ have a reduced ability to regenerate tissue while
  - mice whose activity of $p16^{INK4a}$ is suppressed continue to repair damaged tissue as efficiently as young animals do.
- In mice, eliminating senescent cells (they are high in $p16^{INK4a}$) prevents (in young mice) and partially reverses (in older mice) some of the signs of aging such as cataracts, and loss of adipose tissue and skeletal muscle mass.
- Mice genetically-engineered to express high levels of
  - **telomerase** and
  - tumor suppressor genes (e.g., p53 and $p16^{INK4a}$)

  have substantially-increased life spans and show fewer of the degenerative changes characteristic of aging in their skin and other epithelia. (The need to make these transgenic mice with increased tumor suppressor activity in addition to increased telomerase activity arises because an increased level of telomerase alone elevates the incidence of cancer — killing the animals before any anti-aging effects of telomerase can be measured reliably.)

The role of telomerase deficiency in mammalian aging

Mice whose genes for telomerase have been "knocked out" (either $Tert^{-/-}$ or $Terc^{-/-}$) show many of the degenerative changes associated with aging.

- The number of mitochondria in their cells decreases as does the function of those that remain.
  - Oxygen consumption and ATP production declines.
  - The efficiency of the electron transport chain decreases.
  - This leads to an increased generation of reactive oxygen species (ROS).
- The level of p53 activity increases.
  - mitosis declines
  - apoptosis of cells increases
  - replicative senescence increases
- The anatomy and function of organs such as the liver and heart show the degenerative changes of age.

In the 6 January 2011 issue of **Nature**, Mariela Jaskelioff and her colleagues (many of the same team that found the results described in the previous section) report that reactivation of telomerase in aged mice reverses many signs of aging.

Their experimental animals were another telomerase-deficient strain of mice; that is, mice that couldn't produce telomerase even in those cells — "adult" stem cells and cells of the germline — that normally retain telomerase activity. The mice were made by "knocking-in" a gene that prevents any expression of telomere reverse transcriptase (TERT) unless an activating drug is given to the animal. Without the drug, these mice live half as long as normal, and as they get older, they display many signs of aging:

- their telomeres get shorter leading to chromosome aberrations;
- their cells undergo early replicative senescence;
- almost all their organs — testes, spleen, intestine, brain — show degenerative changes typical of aging.

BUT, if given the activating drug over a four-week period at a time when degenerative changes were already apparent (25-30 weeks), their deterioration stopped and even partially reversed.

- the length of their telomeres increased;
- replicative senescence was delayed;
- their life span was substantially increased;
- their brain, testes, liver, spleen, and intestine escaped the degenerative changes seen in untreated telomerase-deficient mice;
- they produced larger litters than untreated mice;
- there was reduced activation of p53, indicating
  - reduced damage to their genome and
  - reduced apoptosis in their tissues

How would replicative senescence of cells lead to the deterioration in structure and function of the aging tissues (e.g., skin) in which they reside? In tissues, e.g., skin and other epithelia, where mitosis must continue throughout life to replace the cells that are lost, the accumulation of senescent cells — incapable of further mitosis — could leading to the characteristic changes of aging in that tissue.

- One mechanism could be simply the inability of senescent cells to repair the tissue by mitosis.
- However, senescent cells remain active although the genes they express change. Perhaps the proteins they secrete (e.g., collagen-digesting enzymes) cause the aging changes in the tissue where they reside.
- Perhaps it is the senescence of adult stem cells that has the greatest effect on tissue aging. In knockout mice that cannot make the Klotho protein, stem cells and progenitor cells in various tissues undergo senescence and decline in numbers.

An unavoidable tradeoff?

Some of the data so far suggests that efforts to avoid the degenerative changes that come with age (e.g., by increasing cell renewal by means of increased telomerase activity) in the hope of increasing longevity may instead hasten death from cancer while efforts to prevent cancer (e.g., by increasing the activity of tumor suppressor genes) may hasten aging. However, other evidence paints a less-gloomy picture. Mice heterozygous for the p53 tumor suppressor gene ($p53^{+/-}$) develop many cancers when exposed to ionizing radiation. With only a single copy of this tumor suppressor, a single cell is at great risk of losing the remaining copy ("loss of heterozygosity") and starting the growth of a malignant clone. However, if before being irradiated the mice are given resveratrol — to stimulate the production of the anti-aging SIRT1 protein — the incidence of some cancers is reduced and the mice live longer before succumbing to their tumors.

The Accumulation of Genetic Errors

The pros

- Mice given ionizing radiation that damages DNA show early aging.
- Transgenic mice with a defect in the "proofreading" function of the DNA polymerase responsible for copying mitochondrial DNA
- accumulate many mutations in their mitochondrial genes;
- show marked signs of premature aging.
- Cells taken from old mice (and old humans) show slightly elevated levels of somatic mutations and chromosome abnormalities like translocations and aneuploidy. Many of these changes also cause cancer so it is no accident that the incidence of cancer rises with advancing age (graph).
- The hematopoietic stem cells of "knockout" mice deficient in any one of these enzymes needed for genome maintenance
  - *XPD* for nucleotide excision repair (NER)
  - *Ku80* for nonhomologous end joining (NHEJ)
  - *TR* (telomerase RNA) needed for telomere maintenance

  lose their ability to supply the various progenitor cells that produce the white blood cells.
- Most of the hematopoietic stem cells in aged mice show evidence of double-stranded breaks (DSBs) in their chromatin.

- As DSBs form, SIRT1 proteins move from their original locations (at gene promoters) to the locations of the DSBs (where they recruit DNA repair proteins). This shifts the pattern of gene expression to one typical of aging cells.
- Cells taken from old people (and people with premature aging syndromes) show marked reductions in the transcription of some genes, increases in others.

Clues from the Transcriptome of Aging Brains

A group of Harvard researchers reported (in the 26 June 2004 issue of **Nature**) the results of their study of gene expression in the human brain. They extracted the RNA from autopsied brain tissue of 30 people who had died at ages ranging from 26 to 106. They analyzed the RNA with DNA chips looking for the level of activity of some 11,000 different genes (the transcriptome). A clear pattern emerged.

The level of activity of some 400 genes changed over time.

- Gene expression declined in old age for many genes. Some examples:
  - genes encoding proteins involved in synaptic activity in the brain (e.g., learning, memory)
    - NMDA, AMPA, $GABA_A$ receptors
    - calcium-calmodulin-dependent kinase II (CaMKII)
  - genes involved in mitochondrial functions, such as
    - production of ATP (needed for DNA repair)
    - production of damaging reactive oxygen species (**ROS**)

  Detailed examination of some of these down-regulated genes showed that they had suffered DNA damage — more often in their promoters than in their coding regions.
- Gene expression increased in old age for other genes. Some examples:
  - genes involved in inflammation and other immune defenses;
  - genes encoding proteins involved in defense against reactive oxygen species (ROS);
  - genes encoding proteins involved in DNA repair.

The transition from the youthful transcriptome to the transcriptome of the aged brain occurred at varying times from as young as 42 to as old at 73.

A study of individual heart muscle cells in young and old mice (Bahar, R. *et al*., **Nature**, 22 June 2006) showed that the transcriptome of young cells was quite uniform from cell to cell but that of aged cells was highly variable from one cell to another. Variable gene expression from one cell to the next in a single tissue might well lead to defects in the functioning of that tissue.

Clues from Premature Aging Syndromes

Humans suffer from a number of rare genetic diseases that, among other things, produce signs of premature aging, e.g., gray hair, wrinkled skin, and shortened life span. In several cases, the mutated genes are ones that have roles to play in maintaining the integrity of the genome, that is, in DNA repair.

- **Werner's syndrome**. The hair of patients turns gray in their 20s and most die in their late 40s with such signs of age as osteoporosis, cataracts, and atherosclerosis. Even when young, their cells undergo replicative senescence after only ~20 doublings instead of the normal 70 or more. Caused by mutations in *WRN*, which encodes a helicase needed for DNA repair and maintenance of telomeres.
- **Cockayne syndrome** (CS). Caused by mutations in genes needed for DNA repair, especially **transcription-coupled DNA repair**. While these people show only some of the signs of aging, they do have a sharply-reduced life span.
- **Ataxia telangiectasia** (AT). These patients show signs of premature aging. They lack a functioning gene (*ATM*) product needed to detect DNA damage and initiate a repair response.
- **Hutchinson-Gilford progeria syndrome**. Children with this rare disorder show many signs of severe aging by their second birthday and die in their early teens. Caused by mutations in the gene (*LMNA*) for **lamin** the intermediate filament protein that stabilizes the inner membrane of the nuclear envelope. The machinery for DNA replication, transcription, and repair is located at the inner surface of the nuclear envelope, and the cells of these patients have increased DNA damage and other defects in gene expression.

So these syndromes suggest that aging may be the consequence not so much of mutations in general, but of mutations in those genes whose products are essential for the error-free replication, repair and transcription of all genes.

Why is a mouse as old at 2 years as a human at 70

If aging represents the inevitable consequence of a failure of DNA repair, why does it occur so much sooner in some mammals (e.g., mice) than in others (e.g., elephants and humans)?

The answer probably lies in the risk of death from external factors (e.g., predation, starvation, cold) in that species.

As noted above, few small mammals ever age because they die early of external causes. These animals are **r-strategists**, putting their energy into quickly

- reaching sexual maturity
- producing large numbers of offspring that can soon live independently

There is no selective advantage for them to invest in the machinery of efficient DNA repair because they are going to die before mutations become a problem.

Humans, in contrast, are **K-strategists**. They take a long time to reach sexual maturity. They also produce small numbers of young that must be cared for over a long period. Small wonder, then, that evolution in humans (and other long-lived mammals) has selected for genes promoting efficient DNA repair.

The table shows that the efficiency of DNA repair is directly correlated with life span in a variety of mammals.

Correlation between life span and the relative effectiveness of DNA repair in cells of certain mammals. In each case, cells growing in tissue culture were irradiated with ultraviolet light and then the efficiency with which they repaired their DNA was determined. (From the work of R. W. Hart and R. B. Setlow, 1974.)

Species	Average life span, yr	Relative effectiveness of DNA repair
Human	70	50
Elephant	60	47
Cow	30	43
Hamster	4	26
Rat	3	13
Mouse	2	9
Shrew	1	8

Interrelationships

Examining the various factors that have been implicated in the aging process suggests that most —perhaps all — are interrelated.

- Mitochondria disfunction with the production of
- reactive oxygen species (ROS) with their damaging effect on
- DNA and other cell constituents coupled with the
- onset of replicative senescence so that damaged cells can no longer be replaced

may all play important roles. So the factors described above are by no means mutually exclusive.

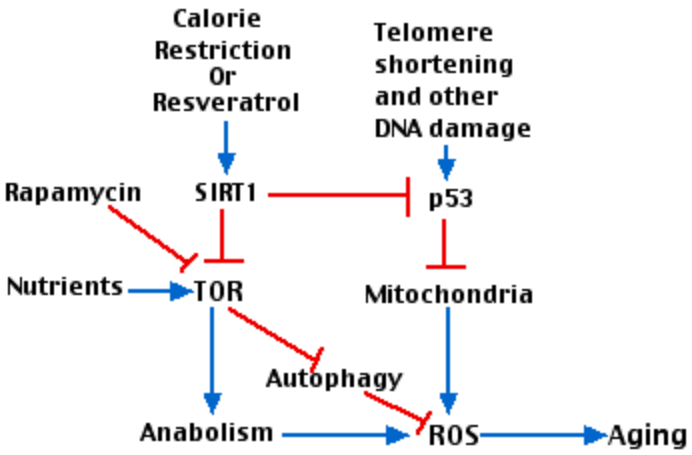


Figure 13.1.2 Factors involved in aging

The above figure attempts to show how various factors involved in aging interact. Key players are

- The gradual shortening of telomeres with repeated cell divisions.
- p53.
- the enzyme designated TOR ("**t**arget **o**f **r**apamycin"). TOR is a kinase that participates in many metabolic pathways in the cell. (It is inhibited by the antibiotic rapamycin that is used as an immunosuppressant).

Stimulatory interactions are shown with blue arrows; inhibitory interactions are shown in red.

Interactions:

- Telomere shortening activates p53 which leads to damaged mitochondria.
- The inefficient electron transport chain in damaged mitochondria produces ROS.
- Abundant nutrients (e.g. amino acids) as well as other growth stimulants activate TOR which promotes anabolism (protein and lipid synthesis) with attendant production of reactive oxygen species (ROS) and aging.
- Calorie restriction, working through SIRT1 inhibits TOR and its downstream effects.
- Inhibition of TOR relieves its inhibition of autophagy allowing the cells to scavenge, for example, damaged mitochondria.
- SIRT1 inhibits p53 activation thus protecting against mitochondrial damage.

Because of the association between telomere shortening and aging, two companies have begun (in 2011) to offer tests of telomere length. How such tests might be useful to the people asking for them remains to be seen.

The Hallmarks of Aging

In the 6 June 2013 issue of **Cell**, an international group of scientists developed a list of 9 features that characterize aging in animals. (This effort to bring order to such a complex subject is reminiscent of earlier papers in the same journal, The Hallmarks of Cancer.)

They expected that each hallmark would meet at least two of three criteria.

- It should be characteristic of normal aging.
- Increased expression of the hallmark should result in faster aging.
- Efforts to reduce expression of the hallmark should prolong a healthy lifespan ("healthspan").

The 9 hallmarks.

1. Genomic Instability
Meeting the criteria:

- Aged cells contain more DNA damage than young ones.
- Agents that increase unrepaired damage to DNA, including chromosomal damage (e.g., aneuploidy), hasten aging.
- Limited evidence that treatments that reduce, for example, chromosome missegregation, prolong healthspan.

2. Telomere Attrition
Meeting the criteria:

- The chromosomes of aged cells have shorter telomeres than those of young cells.
- Telomerase-deficient mice show premature aging.
- Treatments that reactivate telomerase in normal mice delay aging.

3. Epigenetic Alterations
Meeting the criteria:

- The patterns of DNA methylation and histone modifications changes as a mammal ages.
- Mice that are deficient in the sirtuin SIRT6, an enzyme that deacetylates histones, age more rapidly than normal.
- Treatments that increase the activity of sirtuins increase healthspan in mice.

Note

As we humans age, the DNA in our cells accumulates an ever-increasing number of epigenetic changes as measured by the methylation of CpGs. This is true for a wide variety of cell types even those that have been formed recently; that is, the number of epigenetic changes reflects the age of the donor not the age of the cell. The correlation is so good that analysis of these changes in a cell can predict the donor's age sometimes within a matter of months.

4. Loss of Proteostasis
Proteostasis is the homeostasis of the proteome — the proper balance of the synthesis and degradation of proteins in the cell.
Meeting the criteria:

- The clearance of denatured (unfolded) proteins by autophagy and proteasomes, as well as the ability to refolded them with chaperones, all decline with age. The result: toxic protein aggregates that accumulate in aged cells.
- Mutant mice with defective chaperone activity age more quickly.
- Transgenic Drosophila and *C. elegans* that overexpress chaperones have increased life spans.

5. Derugulated Nutrient Sensing
Meeting the criteria:

- The nutrient sensor TOR ("target of rapamycin"), which promotes anabolism, increases during normal aging (and produces obesity, at least in mice).
- Increased activity of TOR accelerates aging in mice.
- Examples:
  - Genetic suppression of TOR signaling extends lifespan in *Drosophila* and *C. elegans*.
  - Calorie Restriction (CR), which inhibits TOR, increases healthspan in all animals in which it has been tested.
  - Rapamycin, which inhibits TOR, extends lifespan in *Drosophila*, *C. elegans*, and mice.

6. Mitochondrial Dysfunction
Meeting the criteria:

- The production and efficiency of mitochondria decreases in aging, otherwise normal, mice.
- Deleterious mutations in mitochondrial DNA and other defects in mitochondrial function all accelerate aging in mice.
- No compelling evidence yet that treatments to improve mitochondrial function increase life span.

7. Cellular Senescence
Meeting the criteria:

- Replicative senescence (cells no longer able to enter the cell cycle) sets in much sooner in the cells of aged animals than it does in young animals.
- Mice engineered to express abnormally-high levels of p53, a protein that blocks entry into the cell cycle, show many signs of premature aging.
- In mice, eliminating senescent cells prevents (in young mice) and partially reverses (in older mice) some of the signs of aging such as cataracts, and loss of adipose tissue and skeletal muscle mass.

8. Stem Cell Exhaustion
Meeting the criteria:

- The proliferative capacity of adult stem cells declines with age in the tissues that have been examined.
- Deliberately exhausting the pool of stem cells in the Drosophila intestine leads to premature aging.
- Transplantation of stem cells from young mice into aged mice improves the degenerative changes of aging and prolongs their life.

9. Altered Intercellular Communication

All cells respond to chemical signals in their environment. These include cytokines secreted by nearby cells (paracrine stimulation).

Meeting the criteria:

- Inflammation in various tissues — mediated by the secretion of a variety of cytokines — increases in the aged.
- Genetically engineered mice that are unable to down-regulate the mRNAs synthesizing pro-inflammatory cytokines show accelerated aging.
- Inhibition of the pro-inflammatory cytokine NF-κB delays aging in mice. Even such a simple anti-inflammatory agent as aspirin seems to prolong life in mice.

Relationships of the Hallmarks

- The first 4 hallmarks appear to represent the initiating events leading to aging.
- Hallmarks 5, 6, and 7 appear to represent damage produced as the cell attempts to respond to the damage caused by the first 4 hallmarks.
- Taken together, hallmarks 1 through 7 produce the aging phenotype seen in hallmarks 8 and 9, which are ultimately responsible for the decline with age in the function of cells and the organism of which they are a part.

An Elixir of Youth?

Despite years of research, only three interventions have been discovered that slow the aging process and/or prolong life.

- **Calorie Restriction** (CR). Works in all animals tested.
- **Rapamycin**. Extends lifespan in mice, *Drosophila*, and *C. elegans* but does not appear to reverse or even halt the degenerative changes of aging.
- **Parabiosis**. When the circulatory system of a young mouse is joined to that of an old mouse (the technique is called parabiosis), various tissues in the old mouse, e.g., its skeletal muscle, cardiac muscle, liver, and central nervous system, become rejuvenated. A promising candidate for mediating this effect is a protein called GDF11 ("Growth Differentiation Factor 11"). (GDF11 is also known as BMP-11). Injections of recombinant GDF11 are almost as effective as parabiosis. GDF11 probably acts by stimulating the activity of stem cells. However, there is no evidence yet that it increases the lifespan of the mice.

Aging in Unicellular Organisms

It used to be thought that many unicellular organisms, such as yeast and bacteria, were immortal; that is, they

- never aged but simply kept dividing to produce new individuals;
- did not have the distinction between germline (immortal) and soma (mortal) that multicellular organisms have.

If true, every time a cell divides, the two daughter cells would be identical in all respects to the parent (symmetrical division).

But at least for yeast and *E. coli*, this is not the case.

Yeast cells do age and, as discussed above, have proved useful for studying the aging process. Placing a single yeast cell on solid medium and removing its daughter (the bud) each time one is produced, it turns out that the number of times the mother cell can form a new bud by mitosis is limited. After producing a bud some 20–30 times, the mother cell shows a number of harmful cellular changes (e.g., defective mitochondria) and dies.

But, at least early in her life, the buds are born with the potential of a full life span. So the mitotic division must be asymmetrical with the properties of the bud different from those of its parent. Several mechanisms by which this occurs have now been demonstrated.

- A diffusion barrier is formed at the neck between the mother cell and her bud. This barrier prevents the passage from the mother into the bud of damaged **nuclear** components, e.g., plasmid-like fragments of DNA produced during the life of the mother. In contrast to the situation in most eukaryotes, in yeast there is no breakdown of the nuclear envelope during mitosis. During anaphase, the nuclear envelope grows and the portion enclosing the daughter chromosomes enters the bud. However, the barrier at the neck keeps all the preexisting nuclear pore complexes (NPCs) within the mother cell and these retain the DNA fragments and perhaps other damaged nuclear components.
- Most of any damaged **cytoplasmic** components, such as proteins denatured by reactive oxygen species (ROS) resulting in the formation of nonfunctional aggregates, become attached to aged mitochondria and the endoplasmic reticulum both of which are retained in the mother cell. Any protein aggregates that do get through are either dissolved by chaperones in the bud or, if that fails, actin filaments in the bud move the aggregates back into the mother cell. This latter mechanism requires the presence of a number of proteins, including a sirtuin found in yeast called Sir2 ("Silent information regulator 2").

Asymmetric division in stem cells.

Stem cells are cells that divide asymmetrically to produce a daughter cell that goes on to differentiate and a daughter cell that remains a stem cell. A number of examples have been found — in *Drosophila* and in mammals — where aging stem cells preferentially deposit their damaged cellular components, e.g. aggregated proteins, in the daughter that will go on to differentiate while keeping undamaged components in the daughter that will remain a stem cell. So like yeast, these stem cells have a mechanism that preferentially protects the "immortal" cell from the inevitable effects of aging.

Aging in *E. coli*

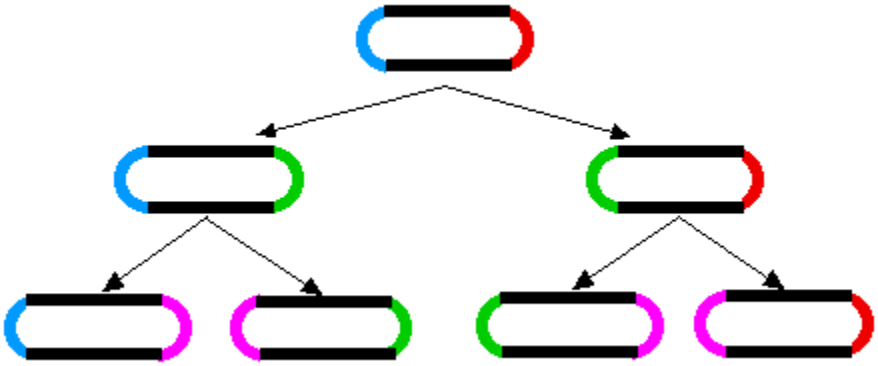


Figure 13.1.3 Formation of new poles during cell division

A similar aging phenomenon has been found in *E. coli*. When *E. coli* divides, a septum forms in the middle of the dividing cell and then the two daughter cells are pinched apart. As the cell wall seals the break, the two daughter cells end up with one "old" end and one newly-formed end. When the two daughters go on to divide, the process is repeated. The original old ends gets passed on from generation to generation (rather like immortal strands of DNA).

The diagram shows how during cell division, two new poles are formed, one in each of the progeny cells (new poles shown in green for the first generation; magenta for the second). The other ends of those cells were formed during a previous division.

It has been shown (Stewart EJ, Madden R, Paul G, Taddei F (2005) Aging and Death in an Organism That Reproduces by Morphologically Symmetric Division. PLoS Biol 3(2): e45 doi:10.1371/journal.pbio.0030045) that the cells that inherit an increasingly old pole exhibit a diminished growth rate, decreased offspring production, and an increased incidence of death.

So it appears that the phenomenon — characteristic of all multicellular organisms — of an aging and mortal soma producing germplasm (sperm and eggs) that starts a new youthful generation may have its counterpart in unicellular organisms. Perhaps no single cell can escape the ravages of time on the integrity of its organelles and the molecules.

Aging in Plants

Annuals and Biennials

Annuals, such as many grasses and "weeds"

- grow vigorously for a period;
- then form flowers followed by fruits.
- Fruiting is followed by a slowing of growth accompanied by physiological and morphological changes such as
  - an increase in the rate of respiration (catabolism)
  - loss of chlorophyll
- These changes constitute aging and end in the death of the plant. Biennials follow the same pattern, but take two years to do it.

  This pattern in clearly programmed in the genes. Even with plentiful moisture, soil minerals, sunlight, and warm temperatures, the plants age and die.

Perennials

The situation is quite different in perennials. Throughout their lives, woody perennials (trees) produce new vascular tissue, leaves, and flowers each year. They do not show marked signs of aging, although their rate of growth may decline over the years. Finally, disease or inability to support their ever-increasing size against wind or snow load lead to their death.



Figure 13.1.4 Bristlecone Pine

This picture (courtesy of Walter Gierasch) is of bristlecone pines (*Pinus longaeva*) growing in the White Mountains of eastern California. Tree-ring analysis shows that some of these trees are almost 5000 years old. But note that **no living cells** in the tree are more than a few years old.

Even so, how have long-lived plants like these avoided the accumulation — over years of DNA replication as their cells divided — of deleterious mutations that would reduce fitness and life span? Perhaps it is because the cells in plant **meristems**, where all growth begins are stem cells which divide slowly and like all stem cells, **asymmetrically**; that is, producing one daughter that will remain a stem cell and one that will begin a phase of **rapid mitosis** and eventually differentiate into the mature tissues of the plant. If (and this is as yet only a speculation) the division of a meristematic stem cell is asymmetric with respect to the segregation of DNA strands; that is, the stem cell retains the immortal strands of DNA while the cell destined to produce more tissues receives the newly-replicated strands, this would provide an additional mechanism to protect the genome as the years go by.

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13.2: Telomeres

Each eukaryotic chromosome consists of a single molecule of DNA associated with a variety of proteins. The DNA molecules in eukaryotic chromosomes are linear; i.e., have two ends. (This is in contrast to such bacterial chromosomes as that in E. coli that is a closed circle, i.e. has no ends.)

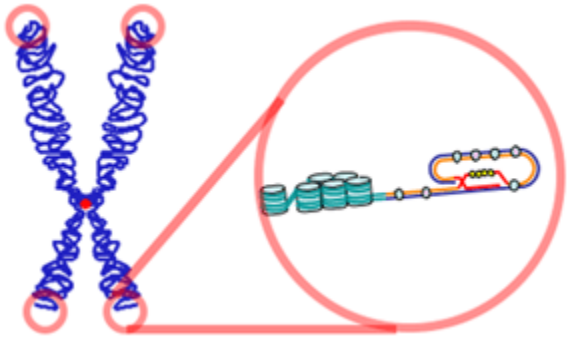


Figure 13.2.1: Telomeres are found at the termini of chromosomes. The end of a telomere inserts back into the main body of the telomere to form a T-loop. (CC BY-SA 3.0; Samulili).

The DNA molecule of a typical chromosome contains a linear array of genes (encoding proteins and RNAs) interspersed with much noncoding DNA. Included in the noncoding DNA are long stretches that make up the centromere and long stretches at the ends of the chromosome, the telomeres. Telomeres are crucial to the life of the cell. They keep the ends of the various chromosomes in the cell from accidentally becoming attached to each other. The telomeres of humans consist of as many as 2000 repeats of the sequence 5' GGTTAG 3'

5'...GGTTAG GGTTAG GGTTAG GGTTAG GGTTAG GGTTAG..3'
3'...CCAATC CCAATC CCAATC CCAATC CCAATC CCAATC..5'

Replication of linear chromosomes presents a special problem

DNA polymerase can only synthesize a new strand of DNA as it moves along the template strand in the 3' –> 5' direction. This works fine for the 3' –> 5' strand of a chromosome as the DNA polymerase can move uninterruptedly from an origin of replication until it meets another bubble of replication or the end of the chromosome. However, synthesis using the 5' –> 3' strand as the template has to be discontinuous. When the replication fork opens sufficiently, DNA polymerase can begin to synthesize a section of complementary strand — called an *Okazaki fragment* — working in the opposite direction. Later, a DNA ligase ("DNA ligase I") stitches the Okazaki fragments together.

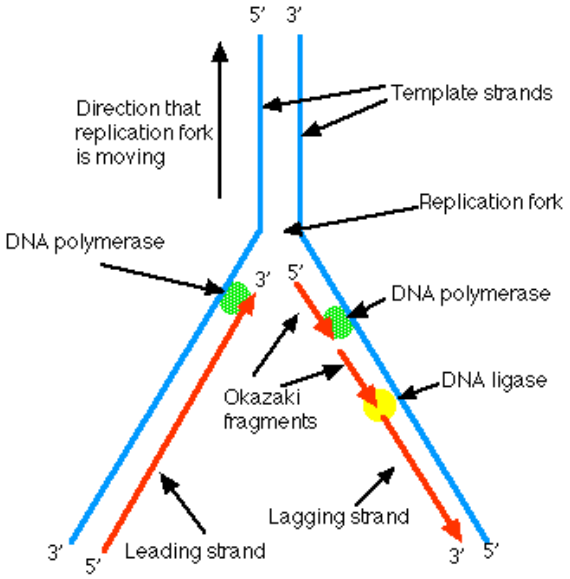


Figure 13.2.2: Okazaki fragments

In Figure 13.2.3, the horizontal black arrows show the direction that the replication forks are moving. Wherever the replication fork of a strand is moving towards the 3' end, the newly-synthesized DNA (red) begins as Okazaki fragments (red dashes). This continues until close to the end of the chromosome. Then, as the replication fork nears the end of the DNA, there is no longer enough template to continue forming Okazaki fragments. So the 5' end of each newly-synthesized strand cannot be completed. Thus each of the daughter chromosomes will have a shortened telomere.

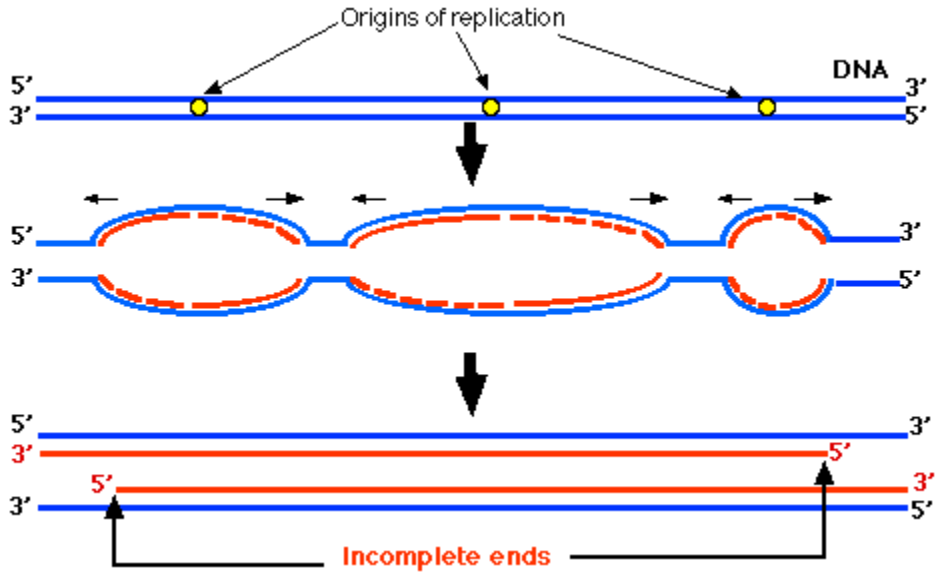


Figure 13.2.3: Direction of Replication fork

It is estimated that human telomeres lose about 100 base pairs from their telomeric DNA at each mitosis. This represents about 16 GGTTAG repeats. At this rate, after 125 mitotic divisions, the telomeres would be completely gone. Is this why normal somatic cells are limited in the number of mitotic divisions before they die out?

Telomeres and Cellular Aging

Telomeres are important so their steady shrinking with each mitosis might impose a finite life span on cells. This, in fact, is the case. Normal (non-cancerous) cells do not grow indefinitely when placed in culture. Cells removed from a newborn infant and placed in culture will go on to divide almost 100 times. Well before the end, however, their rate of mitosis declines (to less than once every two weeks). Were my cells to be cultured (I am 81 years old), they would manage only a couple of dozen mitoses before

they ceased dividing and died out. This phenomenon is called replicative senescence. Could shrinkage of telomeres be a clock that determines the longevity of a cell lineage and thus is responsible for replicative senescence?

Evidence

Some cells do not undergo replicative senescence:

- the cells of the germline (the germplasm)
- unicellular eukaryotes like Tetrahymena thermophila
- stem cells, including "adult" stem cells and cancer stem cells.

It turns out that these cells are able to maintain the length of their telomeres. They do so with the aid of an enzyme telomerase.

Telomerase

Telomerase is an enzyme that adds telomere repeat sequences to the 3' end of DNA strands. By lengthening this strand, DNA polymerase is able to complete the synthesis of the "incomplete ends" of the opposite strand. Telomerase is a ribonucleoprotein. Its single snoRNA molecule — called TERC ("TElomere RNA Component") — provides an CCAAUC (in mammals) template to guide the insertion of GGTTAG. Its protein component — called TERT ("TElomere Reverse Transcriptase") — provides the catalytic action. Thus telomerase is a reverse transcriptase; synthesizing DNA from an RNA template.

Telomerase is generally found only in the cells of the germline, including embryonic stem (ES) cells; unicellular eukaryotes like Tetrahymena thermophila; and some, perhaps all, "adult" stem cells (including cancer stem cells) and "progenitor" cells enabling them to proliferate. When normal somatic cells are transformed in the laboratory with DNA expressing high levels of telomerase, they continue to divide by mitosis long after replicative senescence should have set in. And they do so without any further shortening of their telomeres. This remarkable demonstration (reported by Bodnar et. al. in the 16 January 1998 issue of Science) provides the most compelling evidence yet that telomerase and maintenance of telomere length are the key to cell immortality.

Telomere Deficiency Syndromes

A number of rare human diseases are caused by mutations in *TERT* or *TERC* or several other genes involved in telomere maintenance. The severity of the disease, the organs it affects, and the age of onset vary widely. But all are characterized by abnormally short telomeres. Patients with telomere deficiency are also at increased risk of developing cancer.

Telomerase and Cancer

The crucial feature that distinguishes a cancer from normal tissue is its ability to grow indefinitely. Most (85–90%) cancers express telomerase — at least in the population of cancer stem cells that divide uncontrollably causing the tumor to grow. Perhaps agents that prevent the expression of the gene for telomerase — or prevent the action of the enzyme — will provide a new class of weapons in the fight against cancer. But if telomerase activity — however brief — is essential for all cells, we had better be careful, and if lack of telomerase hastens replicative senescence, it may also hasten the aging of the tissues that depend on newly-formed cells for continued health — a tradeoff that may not be worth making.

Telomerase and Transplanted Cells

One approach to gene therapy it to remove cells from the patient, transform them with the gene for the product that the patient has been unable to synthesize, return them to the patient. One problem with this approach is that the cells — like all normal somatic cells — are mortal. After a series of mitotic divisions, they die out. That is the reason the children described in the link above required periodic fresh infusions of their transformed T cells.

What if their cells could be transformed not only with the therapeutic gene, but also with an active telomerase gene? This should give them an unlimited life span. But if cancer cells regain the ability to make telomerase, might not the reverse be true; that cells transformed with an active telomerase gene might become cancerous? Perhaps not. The cells described by Bodnar et. al. in the 16 January 1998 issue of Science have continued to grow in culture and have been subjected to a number of tests to see if they have acquired any properties of cancer cells in culture.

The results are encouraging. While these cells continue to divide indefinitely as cancer cells do,

- They still show contact inhibition as normal cells do when grown in culture.
- They do not grow into tumors when injected into immunodeficient mice (as cancer cells do).
- They are still fussy about their diet — unable to grow on the simple media that supports cancer cells in culture.
- They still retain a normal karyotype; something that cancer cells seldom do.

However, studies with whole animals — transgenic mice that express abnormally high levels of TERT — reveal that they do suffer an elevated incidence of cancer.

Telomeres and Cloning

The now-famous sheep Dolly was cloned using a nucleus taken from an adult sheep cell that had been growing in culture. The cell donor was 6 years old, and its cells had been growing in culture for several weeks. What about Dolly's telomeres? Analysis of telomere length in Dolly's cells reveals that they were only 80% as long as in a normal one-year-old sheep. Not surprising, since the nucleus that created Dolly had been deprived of telomerase for many generations. Two other sheep — cloned from embryonic, not adult, cells — also had shortened telomeres although not as short as Dolly's. Perhaps the length of time the cells spent in culture before they were used accounts for this.



Dolly. (Cc BY-SA 2.0 Toni Barros).

Does this mean that Dolly is doomed to a shortened life? She seemed healthy at first and even had babies of her own. But medical problems — probably unrelated to her telomeres — ended with her being euthanized at a relatively young age. But her short telomeres do add another question to the debate about cloning mammals from adult cells.

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UNIT 14: EMBRYONIC DEVELOPMENT AND ITS REGULATION

Embryogenesis is the process by which the embryo forms and develops. In mammals, the term refers chiefly to early stages of prenatal development, whereas the terms fetus and fetal developmentdescribe later stages. Embryogenesis starts with the fertilization of the egg cell (ovum) by a sperm cell, (spermatozoon).

14.1: Embryonic Development

14.2: Frog Embryology

14.3: Cleavage

14.4: The Organizer

14.5: Segmentation - Organizing the Embryo

14.6: Homeobox Genes

14.7: Stem Cells

14.8: Embryonic Stem Cells

14.9: Germline vs. Soma

14.10: Regeneration

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14.1: Embryonic Development

In animals, one can usually distinguish 4 stages of embryonic development.

- Cleavage
- Patterning
- Differentiation
- Growth

Cleavage

Mitosis and cytokinesis of the zygote, an unusually large cell, produces an increasing number of smaller cells, each with an exact copy of the genome present in the zygote. However, the genes of the zygote are not expressed at first. The early activities of cleavage are controlled by the mother's genome; that is, by mRNAs and proteins she deposited in the unfertilized egg. In humans, the switch-over occurs after 4–8 cells have been produced; in frogs not until thousands of cells have been produced. Cleavage ends with the formation of a **blastula**.

Patterning

During this phase, the cells produced by cleavage organize themselves in layers and masses, a process called **gastrulation**. The pattern of the future animal appears:

- front to rear (the **anterior-posterior** axis)
- back side and belly side (its **dorsal-ventral** axis)
- left and right sides.

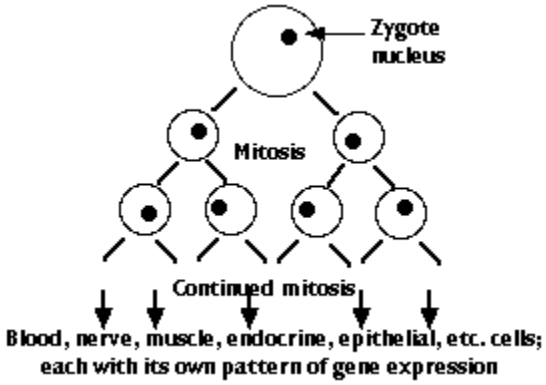


Figure 14.1.1 Zygote Mitosis

The genome of the zygote contains all the genes needed to make the hundreds of different types of cells that will make up the complete animal. There are two major categories of these genes:

- "housekeeping" genes = those that encode the RNAs and proteins needed by all kinds of cells. Examples:
  - genes for tRNAs, rRNAs
  - genes encoding the enzymes of glycolysis.
- tissue-specific genes = those that encode mRNAs and hence proteins that are used by one or a few specific kinds of cell. Examples:
  - genes for hemoglobin expressed in the precursors of red blood cells
  - the gene for insulin expressed in the beta cells of the islets of Langerhans

However, every cell descended from the zygote has been produced by mitosis and thus contains the complete genome of the organism (with a very few exceptions).

Two pieces of evidence:

- Dolly - Dolly is the sheep that was formed by inserting a nucleus from a single cell of an **adult** sheep into an enucleated sheep egg. She proves that the cell from the adult had lost none of the genes needed to build all the tissues of a sheep.
- Spemann's egg-tying experiments - Many years earlier, the German embryologist Hans Spemann demonstrated the same truth. He used strands of baby hair to tie loops around fertilized salamander eggs. Although the egg half with the nucleus began cleaving normally, the other side did not begin cleavage until a nucleus finally slipped through the knot. So long as the egg was tied so that both halves contained some of the gray crescent, the second half began normal cleavage and ultimately produced a second tadpole (right). Even after 5 mitotic division of the zygote nucleus (the 32-cell stage), the entire genome was still available in each descendant nucleus.

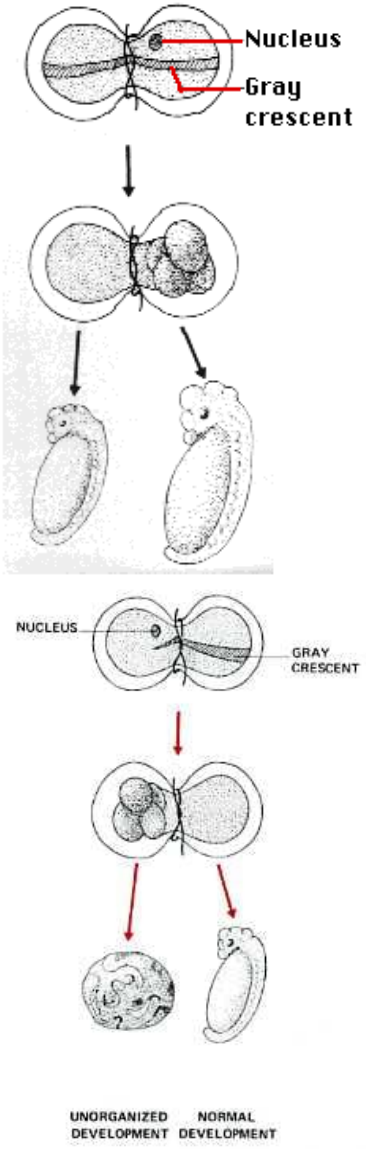


Figure 14.1.3 Frog embryo courtesy L. M. Beidler

1. A fertilized egg is much larger than the normal cells of an animal's body. Some (e.g., a hen's egg) are truly huge. The frog egg has a volume 1.6 millions times larger than a typical frog cell. The photo is of a 16-cell frog embryo. This mass of cells is no larger than the original egg. The eggs of mammals are smaller, but even they are larger than their descendant cells will be.
2. The cytoplasm of the fertilized egg is **not homogeneous**. It contains gradients of mRNAs and proteins. These are the products of the mother's genes and were deposited in the egg by her.
3. Cleavage of the fertilized egg partitions it into thousands of cells of normal size. Each contains a nucleus descended from the zygote nucleus.
4. But each nucleus finds itself partitioned off in cytoplasm containing a particular mix of mRNAs and proteins.

5. When the frog blastula has produced some 4,000 cells, transcription and translation of its nuclear genes begins (and the mother's mRNA molecules, that up to now have been the source of all protein synthesis, are destroyed).
6. The genes that are expressed by the nucleus in a given cell are regulated by the molecules, mostly protein transcription factors and microRNAs (miRNAs), found in the cytoplasm surrounding that nucleus.
7. Once a cell-specific pattern of gene expression is launched, that cell may release molecules that regulate the genes of nearby cells.
8. In this way, the foundation is laid for the building of an organism with hundreds of types of differentiated cells — each in its correct location and performing its correct functions.

Xenopus

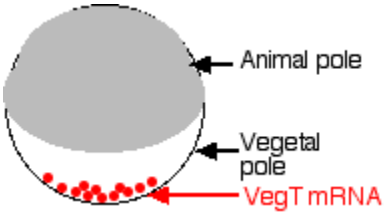


Figure 14.1.4 Vegetal Pole

- During egg formation, molecules of mRNA encoding the protein VegT are deposited at the vegetal pole of the cell.
- Cells that form there during cleavage translate the mRNA into the **VegT** protein.
- VegT is a transcription factor that turns on genes that produce members of the transforming growth factor-beta (TGF-β) family (e.g., **activin**).
- These proteins are needed for cells to start down the path to becoming **mesoderm**.
- Some of those cells will, in turn, become the **Spemann organizer**.
- Later, the Spemann organizer will secrete molecules that induce the ectodermal cells above them to develop into the tissues of the brain and spinal cord.

Demonstration

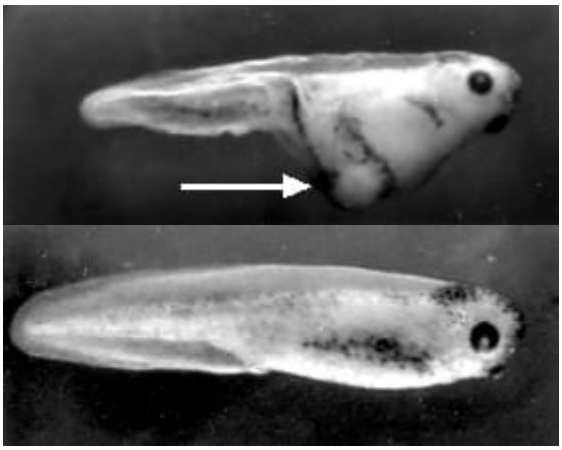


Figure 14.1.7 Double posterior larva

Inject the anterior of the fertilized egg with nanos mRNA. The result: another double-posterior larva.

Make female fruit flies that are transgenic for a recombinant gene containing:

- the gene for **nanos**
- coupled to the **3´ anterior-directing signal of the bicoid** gene.

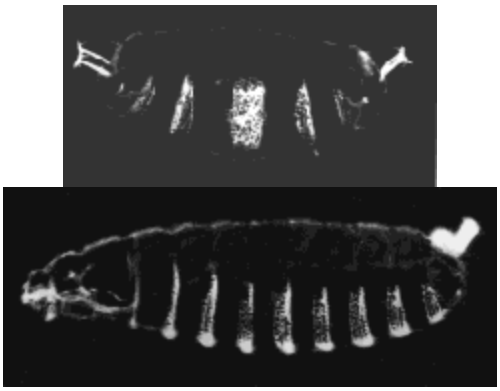


Figure 14.1.8 Larva images Elizabeth Gavis and Ruth Lehmann

A normal larva is shown on the right. The bright object at the right end of the normal larva and at both ends of the double posterior larva is the tip of the tail. These micrographs are courtesy of Elizabeth Gavis and Ruth Lehmann, in whose lab the third demonstration was performed.

The Mud Snail

The mud snail, **Ilyanassa obsoleta**, is a small gastropod that lives in mud flats along the Atlantic coast.

Like other **protostomes**, cleavage of the zygote produces daughter cells that are already committed to their fate. In other words, even as early as the two-cell stage, the cells are no longer totipotent. Unlike humans and other **deuterostomes**, then, identical twins cannot form.

In the 12 December 2002 issue of **Nature**, J. David Lambert and Lisa Nagy reported another mechanism by which two daughter cells become committed to different fates even though they have inherited the same genome.

They traced the distribution in the cells of early embryos of the **messenger RNAs** (mRNAs) encoding 3 proteins that are known to be important in the development of other animals such as Xenopus and Drosophila.

- *IoEve*, which is **I**lyanassa **o**bsoleta's version of ***even-skipped*** (***eve***) in Drosophila;
- ***IoDpp***, which is the snail's version of
  - ***decapentaplegic*** (***dpp***) in Drosophila and the genes encoding
  - **b**one **m**orphogenic **p**roteins (**BMP2** and **BMP4**) in vertebrates
- ***IoTld***, which encodes the snail's version of a protein called tolloid in Drosophila.

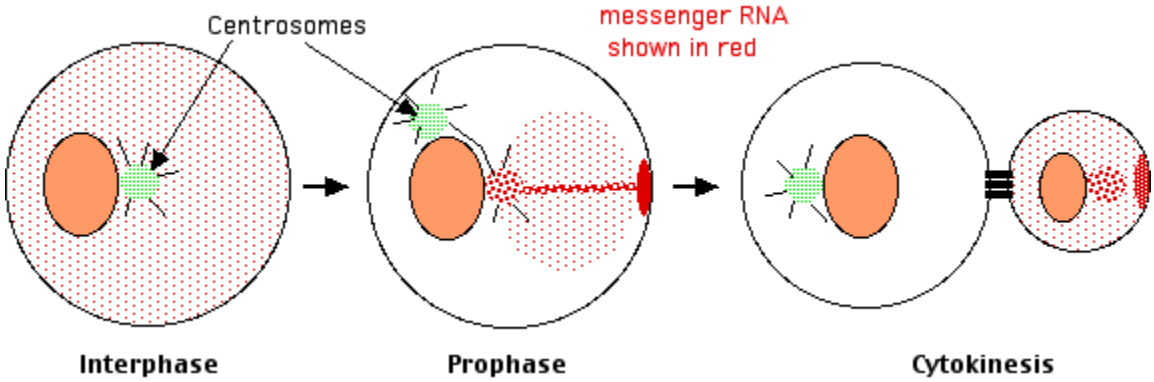


Figure 14.1.9 Duplication of centrosomes before mitosis

Lambert and Nagy found that

- in interphase the messenger RNAs were distributed diffusely throughout the cytosol, but
- as the cell got ready for cleavage, the mRNAs collected at **only one** of the now pair of centrosomes. They were collected there by traveling along the microtubules that radiate out from the centrosome.

- As cleavage continued, the mRNAs moved from the centrosome to a spot on the inner surface of the plasma membrane. They got there by traveling along actin filaments.
- At cytokinesis, this patch of accumulated mRNAs was incorporated exclusively into the smaller daughter cell.

Centrosome sorting (of proteins in this case) also plays a role in determining whether embryonic cells of ***Caenorhabditis elegans*** remain in the **germline** or become the **somatic** cells of the worm.

What comes next?

Development in Xenopus and Drosophila passes through three rather different (although often overlapping) phases:

- Establishing the main axes (anterior-posterior; dorsal-ventral; left-right). This is done by gradients of mRNAs and proteins encoded by the mother's genes and placed in the egg by her. It has been discussed here.
- Establishing the main body parts such as the notochord and central nervous system in vertebratesand the segments in DrosophilaThese are run by genes of the zygote itself.
- Filling in the details; that is, building the various organs of the animal.

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14.2: Frog Embryology

The Egg

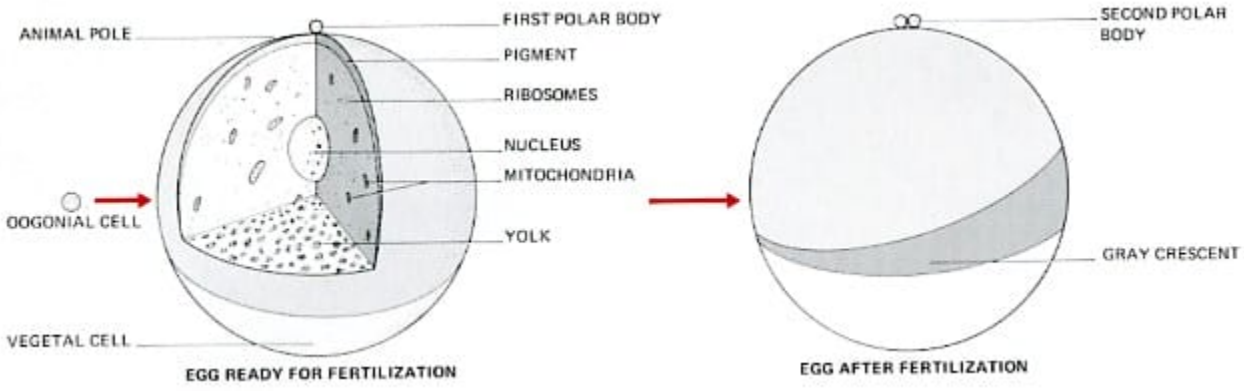


Figure 14.2.1 Frog Egg

The frog egg is a huge cell; its volume is over 1.6 million times larger than a normal frog cell. During embryonic development, the egg will be converted into a tadpole containing millions of cells but containing the same amount of organic matter.

- The upper hemisphere of the egg — the **animal pole** — is dark.
- The lower hemisphere — the **vegetal pole** — is light.
- When deposited in the water and ready for fertilization, the haploid egg is at metaphase of meiosis II.

Fertilization

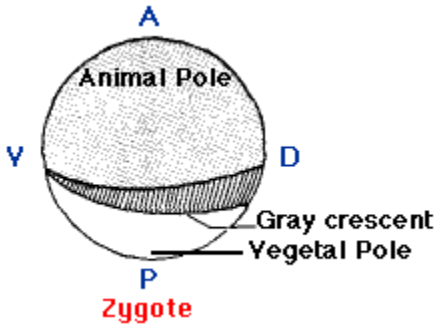


Figure 14.2.2 Frog Zygote

Entrance of the sperm initiates a sequence of events:

- Meiosis II is completed.
- The cytoplasm of the egg rotates about 30 degrees relative to the poles.
- In some amphibians (including Xenopus), this is revealed by the appearance of a light-colored band, the **gray crescent**.
- The gray crescent forms opposite the point where the sperm entered.
- It foretells the future pattern of the animal: its dorsal (**D**) and ventral (**V**) surfaces; its anterior (**A**) and posterior (**P**); its left and right sides.
- The haploid sperm and egg nuclei fuse to form the diploid zygote nucleus.

Cleavage

The zygote nucleus undergoes a series of mitoses, with the resulting daughter nuclei becoming partitioned off, by cytokinesis,

in separate, and ever-smaller, cells. The **first cleavage** occurs shortly after the zygote nucleus forms. A furrow appears that runs longitudinally through the poles of the egg, passing through the point at which the sperm entered and bisecting the gray crescent. This divides the egg into two halves forming the **2-cell stage.** The **second cleavage** forms the **4-cell stage**. The cleavage furrow again runs through the poles but at right angles to the first furrow. The furrow in the **third cleavage** runs horizontally but in a plane closer to the animal than to the vegetal pole. It produces the **8-cell stage**.

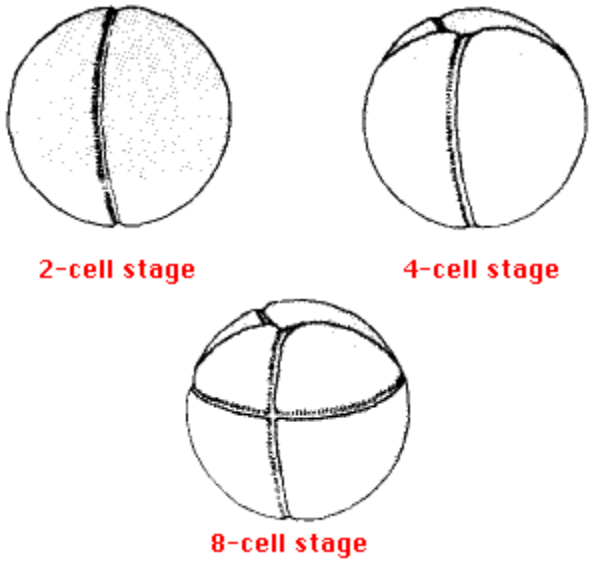


Figure 14.2.3 Various stages of cleavage in a frog zygote

The next few cleavages also proceed in synchrony, producing a 16-cell and then a 32-cell embryo. However, as cleavage continues, the cells in the animal pole begin dividing more rapidly than those in the vegetal pole and thus become smaller and more numerous. By the next day, continued cleavage has produced a hollow ball of thousands of cells called the **blastula**. A fluid-filled cavity, the **blastocoel**, forms within it.

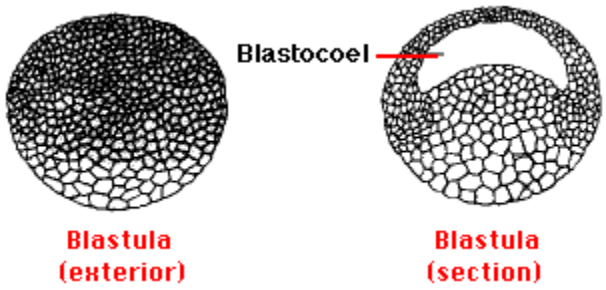


Figure 14.2.4 Frog Bastula

During this entire process there has been no growth of the embryo. In fact, because the cells of the blastula are so small, the blastula looks just like the original egg to the unaided eye. Not until the blastula contains some 4,000 cells is there any transcription of zygote genes. All of the activities up to now have been run by gene products (mRNA and proteins) deposited by the mother when she formed the egg.

Gastrulation

The start of gastrulation is marked by the pushing inward ("invagination") of cells in the region of the embryo once occupied by the middle of the gray crescent.

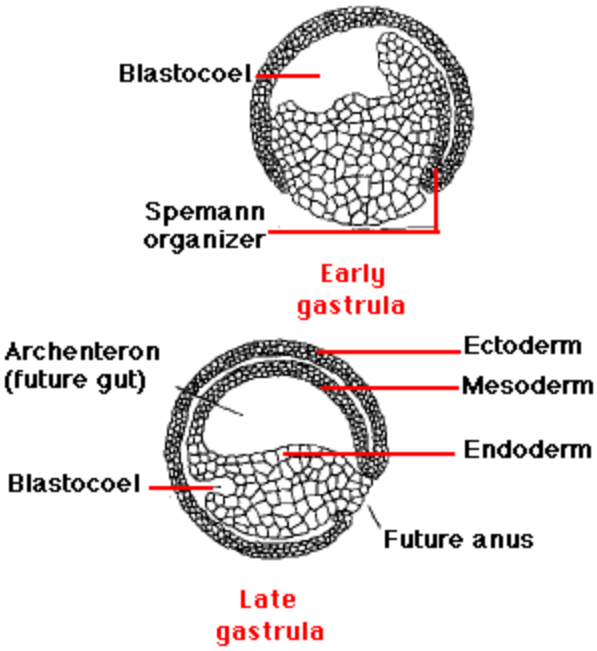


Figure 14.2.5 Frog gastrula

This produces an opening (the **blastopore**) that will be the future anus. a cluster of cells that develops into the **Spemann organizer** (named after one of the German embryologists who discovered its remarkable inductive properties).

As gastrulation continues, three distinct **"germ layers"** are formed:

- ectoderm
- mesoderm
- endoderm

Each of these will have special roles to play in building the complete animal. Some are listed in the table.

Germ-layer origin of various body tissues

Ectoderm	Mesoderm	Endoderm
skin	notochord	inner lining of gut, liver, pancreas
brain	muscles	inner lining of lungs
spinal cord	blood	inner lining of bladder
all other neurons	bone	thyroid and parathyroid glands
sense receptors	sex organs	thymus

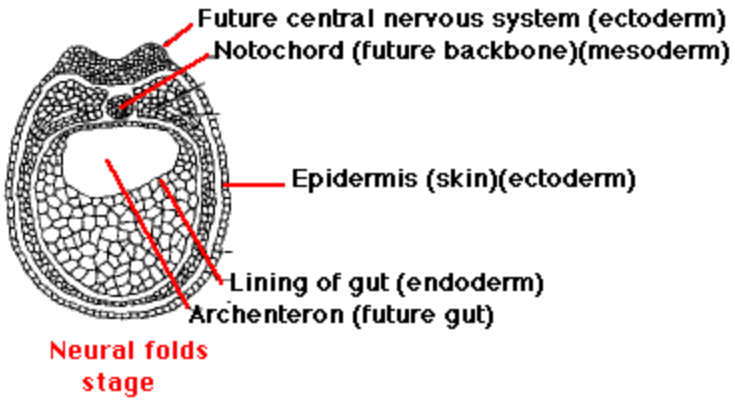


Figure 14.2.6 Frog neural folds

The Spemann organizer (mostly mesoderm) will develop into the **notochord**, which is the precursor of the backbone and induce the ectoderm lying above it to begin to form neural tissue instead of skin. This ectoderm grows up into two longitudinal folds, forming the **neural folds** stage. In time the lips of the folds fuse to form the **neural tube**. The neural tube eventually develops into the brain and spinal cord.

Differentiation

Although the various layers of cells in the frog gastrula have definite and different fates in store for them, these are not readily apparent in their structure. Only by probing for different patterns of gene expression (e.g., looking for tissue-specific proteins) can their differences be detected. In due course, however, the cells of the embryo take on the specialized structures and functions that they have in the tadpole, forming **neurons**, **blood cells**, **muscle cells**, **epithelial cells**, etc., etc.

Growth

At the time the tadpole hatches, it is a fully-formed organism. However, it has no more organic matter in it than the original frog egg had. Once able to feed, however, the tadpole can grow. It gains additional molecules with which it can increase the number of cells that make up its various tissues.

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14.3: Cleavage

Cleavage refers to the early cell divisions that occur as a fertilized egg begins to develop into an embryo.

Holoblastic Cleavage

In eggs that contain no (mammals) or only moderate amounts (frog) of yolk, cytokinesis divides the cells completely. The figure shows the results of the first two cleavages in the frog embryo.

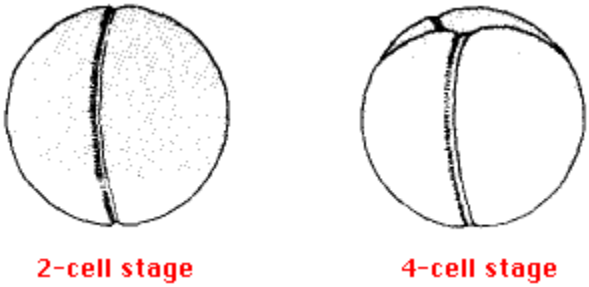


Figure 14.3.1: First two cleavages in a frog embryo

Meroblastic Cleavage

In eggs that contain a large amount of yolk, cytokinesis does not divide the egg completely.

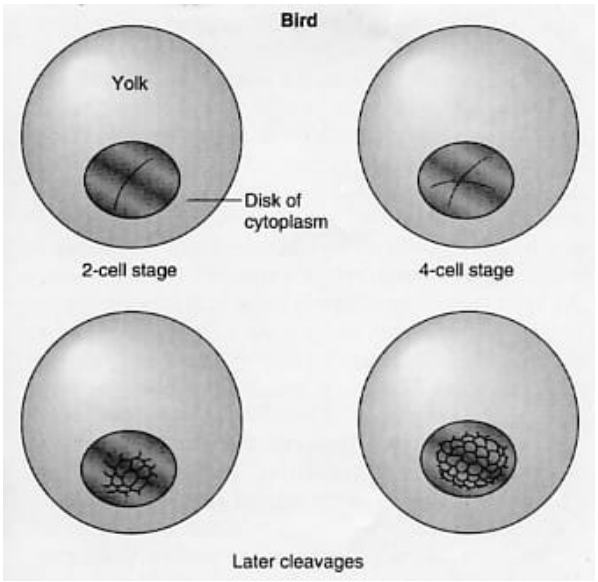


Figure 14.3.2: Bird cleavage

The hen's egg consists of just a tiny patch of cytoplasm resting on the surface of a large ball of yolk (the "white" of the egg is noncellular accessory protein). When the first cleavages occur in the hen's egg, the cleavage furrows do not continue down through the mass of yolk. Therefore, each of the cells produced in the earliest stages is bound on the top and on the sides by a plasma membrane, but the bottom of the cell is in direct contact with yolk.

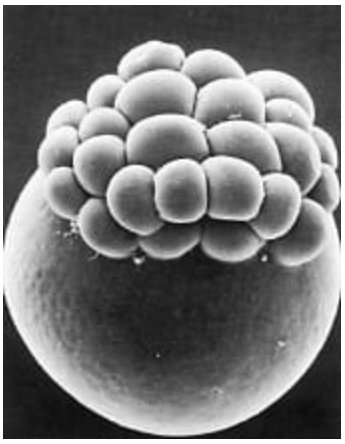


Figure 14.3.3: Zebrafish cleavage

This type of meroblastic cleavage is also found in the eggs of fish, reptiles, and 4 species of mammals — the monotremes. This photo, courtesy of H. W. Beames and Richard G. Kessel, shows the zebrafish (Danio) embryo at the 32-cell stage. Note that the cleavage furrows have not continued down through the yolk of the egg.

Insects use a different type of meroblastic cleavage.

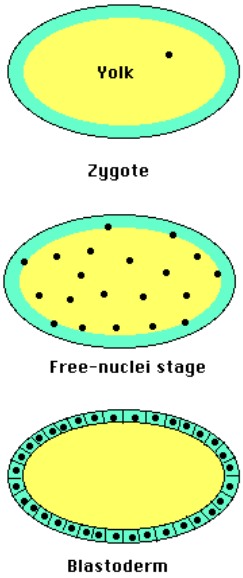


Figure 14.3.4: Insect cleavage

The yolk of the eggs of insects is concentrated in the center of the egg. The daughter nuclei produced by mitosis of the zygote nucleus remain suspended within the single egg compartment. After several thousand nuclei have been produced, they migrate to the cytoplasm-rich margin of the egg. Only then does a plasma membrane form around each one.

What does cleavage accomplish in the development of the organism? First, it provides a stockpile of cells out of which the embryo will be constructed. Second, cleavage establishes a normal relationship between the nucleus and the volume of cytoplasm it regulates (and which in turn regulates it). Even small eggs are enormous when compared with other kinds of cells. The volume of the frog egg is about 1.6 million times larger than that of a normal frog cell. But it, too, contains only a single nucleus. During cleavage, thousands of new nuclei are produced by mitosis all of which finally end up in a cell of normal dimensions. The frog blastula, with its thousands of cells is no larger than the original fertilized egg.

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14.4: The Organizer

In the embryonic development of a zygote, gradients of mRNAs and proteins, deposited in the egg by the mother as she formed it, give rise to cells of diverse fates despite their identical genomes. But is the embryo fully patterned in the fertilized egg? It is difficult to imagine that the relatively simple gradients in the egg could account for all the complex migration and differentiation of cells during embryonic development. And, in fact, the answer is no. However, once these gradients have sent certain cells along a particular path of gene expression, the stage is set for those cells to begin influencing nearby cells to become increasingly diversified.

In other words, *cell-intrinsic signals* (established between a nucleus and the particular cytoplasmic environment that cleavage has placed it in) lay the foundation for *cell-cell interactions* to further guide the cells of the embryo to assume their proper position in the embryo and to differentiate into their final specialized form and function.

Cell-cell interactions could — and probably do — occur in several ways:

- diffusion of a signaling molecule out of one cell and into other cells in the vicinity;
- diffusion of a signaling molecule from one cell into an adjacent cell that then secretes the same molecule to diffuse to the next cell and so on (a "cell-relay" mechanism);
- extension of projections from the plasma membrane of one cell until they make **direct contact** with nearby cells. This enables proteins embedded in the plasma membrane to serve as signaling molecules.

The Spemann Organizer

In 1924, the Ph.D. student Hilde Mangold working in the laboratory of German embryologist Hans Spemann performed an experiment that demonstrated that the pattern of development of cells **is** influenced by the activities of other cells and stimulated a search, which continues to this day, for the signals at work. Spemann and Mangold knew that the cells that develop in the region of the gray crescent migrate into the embryo during gastrulation and form the notochord (the future backbone; made of **mesoderm**). She cut out a piece of tissue from the gray crescent region of one newt gastrula and transplanted it into the ventral side of a second newt gastrula. To make it easier to follow the fate of the transplant, she used the embryo of one variety of newt as the donor and a second variety as the recipient.

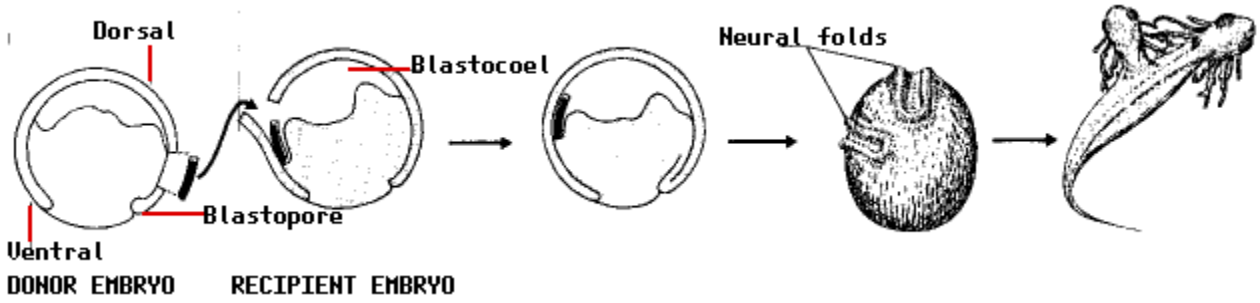


Figure 14.4.1: Spemann Experiment

The remarkable results:

- the transplanted tissue developed into a second notochord
- **neural folds** developed above the extra notochord
- these went on to form a second central nervous system (portions of brain and spinal cord) and eventually
- a two-headed tadpole.

The most remarkable finding of all was that the neural folds were built from recipient cells, not donor cells. In other words, the transplant had altered the fate of the overlying cells (which normally would have ended up forming skin [epidermis] on the side of the animal) so that they produced a second head instead!

Spemann and Mangold used the term induction for the ability of one group of cells to influence the fate of another. And because

of the remarkable inductive power of the gray crescent cells, they called this region the organizer. Ever since then, vigorous searches have been made to identify the molecules liberated by the organizer that induce overlying cells to become nerve tissue. One candidate after another has been put forward and then found not to be responsible. Part of the problem has been that not until just recently has it become clear that the organizer does NOT induce the central nervous system but instead it prevents signals originating from the ventral side of the blastula from inducing skin (epidermis) there.

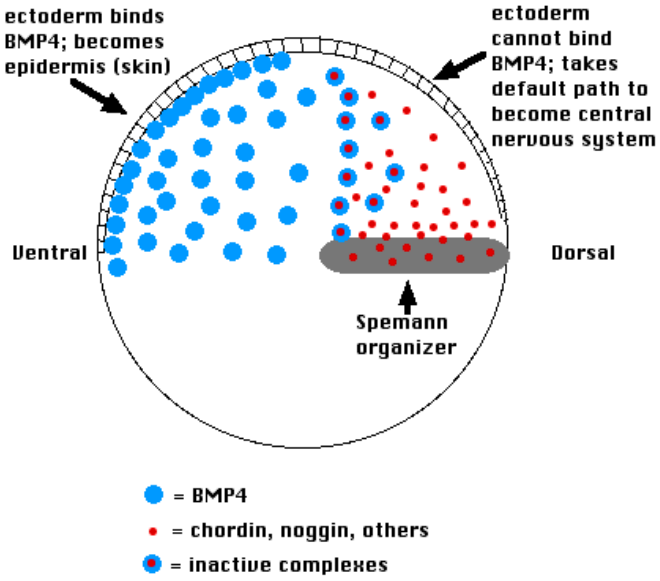


Figure 14.4.2: Spemann organizer

This is how it works:

- Cells on the **ventral** side of the blastula secrete a variety of proteins such as **b**one **m**orphogenetic **p**rotein-4 (**BMP-4**)
- These **induce** the **ectoderm** above to become **epidermis**.
- If their action is blocked, the ectodermal cells are allowed to follow their **default pathway**, which is to become nerve tissue of the brain and spinal cord.
- The Spemann **organizer blocks the action of BMP-4** by secreting molecules of the proteins **chordin** and **noggin**
- Both of these physically bind to BMP-4 molecules in the extracellular space and thus prevent BMP-4 from binding to receptors on the surface of the overlying ectoderm cells.
- This allows the ectodermal cells to follow their intrinsic path to forming neural folds and, eventually, the brain and spinal cord.

In the Spemann/Mangold experiment, transplanting an organizer to the ventral side provided a second source of chordin. This blocked BMP-4 binding to the overlying ectoderm and thus changed the fate of those cells to forming a second central nervous system rather than skin.

What Organizes the Organizer?

Protein synthesis by the cells of the organizer requires transcription of the relevant genes (e.g., *chordin*). Expression of organizer genes depends first on Wnt transcription factors. Their messenger RNAs were deposited by the mother in the vegetal pole of the egg. After fertilization and formation of the gray crescent, they migrated into the gray crescent region (destined to become the organizer) where they were translated into Wnt protein.

Its accumulation on the dorsal side of the embryo unleashes the activity of **Nodal** — a member of the Transforming Growth Factor-beta (TGF-β) family. Nodal induces these dorsal cells to begin expressing the proteins of Spemann's organizer.

A Tail Organizer

One of the distinguishing features of vertebrates is their tail, which extends out behind the anus. French researchers have reported (in the 24 July 2003 issue of **Nature**) their discovery of a tail "organizer", that is, a cluster of cells in the embryo that induces nearby cells to contribute to the formation of the tail. They worked with the zebrafish, *Danio rerio* (which also has a head

organizer like that of newts). They removed tiny clusters of cells from the ventral part of the blastula (a region roughly opposite where the Spemann-like organizer forms) and transplanted this into a region of the host embryo that would normally form flank. The result: a second tail.

Using a fluorescent label, they were able to show that the extra tail was made not only from descendants of the transplanted cells but also from host cells that would normally have made flank. Three proteins were essential:

- a Wnt protein (establishes the anterior-posterior axis in all bilaterians)
- BMP (establishes the dorsal-ventral axis in all bilaterians)
- Nodal (establishes the left-right axis in all bilaterians)

Patterning the central nervous system in Drosophila

Remarkably, it turns out that proteins similar in structure to the bone morphogenetic proteins and also to chordin are found in Drosophila. The role of **BMP-4** is taken by a related protein encoded by the *decapentaplegic* gene (*dpp*) and the role of chordin is taken by a related protein called SOG encoded by the gene called *short gastrulation*.

In fact, these proteins and their mRNAs are completely interchangeable! An injection of the mRNAs for BMP-4 or chordin into the blastoderm of the Drosophila embryo can replace the function of DPP and SOG respectively, and conversely, injections of mRNA for DPP or SOG into the Xenopus embryo mimics the functions of BMP-4 and chordin respectively.

Table 14.4.1*: A selection of antagonistic pairs of proteins that guide the patterning of the embryo.*

Xenopus		blocked by chordin
	and also by noggin	
Drosophila	Decapentaplegic (DPP)	blocked by short gastrulation (SOG)
		and also by a noggin homolog?

Dorsal vs Ventral Nerve Cords

Although their actions are similar, the distribution of these proteins in Drosophila differs from that in Xenopus (as well as in mammals and other vertebrates). In Drosophila, **DPP** is produced in the **dorsal** region of the embryo and **SOG** is produced in the **ventral** region.

However, their actions on overlying cells are the same as in Xenopus; that is, the SOG protein prevents the DPP protein from blocking the formation of the central nervous system. The result in Drosophila is that its central nervous system forms on the **ventral** side of the embryo, not on the dorsal! And, you may remember that one of the distinguishing traits of all arthropods (insects, crustaceans, arachnids) as well as many other invertebrates, such as the annelid worms, is a **ventral nerve cord**. Chordates, including all vertebrates, have a dorsal (spinal) nerve cord.

We're halfway done!

Xenopus development (and probably that of animals in general) passes through three rather different (although often overlapping) phases:

- establishing the main axes (dorsal-ventral; anterior-posterior; left-right). This is done by gradients of mRNAs and proteins encoded by the mother's genes and placed in the egg by her.
- establishing the main body parts such as
  - the notochord and central nervous system in vertebrates (discussed here and also described in Fro.g Embryology)
  - and the segments in Drosophila

  These are run by genes of the zygote itself.
- filling in the details; that is, building the various organs of the animal. (Our examples will include the wings, legs, and eyes of Drosophila.)

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14.5: Segmentation - Organizing the Embryo

Insects, like all arthropods, are segmented. The body of *Drosophila melanogaster* is built from 14 segments:

- 3 segments make up the **head** with its antennae and mouth parts.
- 3 segments make up the **thorax**. Each thoracic segment has a pair of legs (insects are the six-legged creatures). In Drosophila (and other flies), the middle thoracic segment carries a single pair of wings; the hind segment a pair of halteres.
- 8 **abdominal segments**.

What signals guide segment formation? The process begins with the gradients of messenger RNA (mRNA) that the mother deposited in her egg before it was fertilized. Shortly after fertilization, these are translated into their proteins with a gradient of **bicoid** diminishing from anterior to posterior and a gradient of **nanos** diminishing from posterior to anterior.

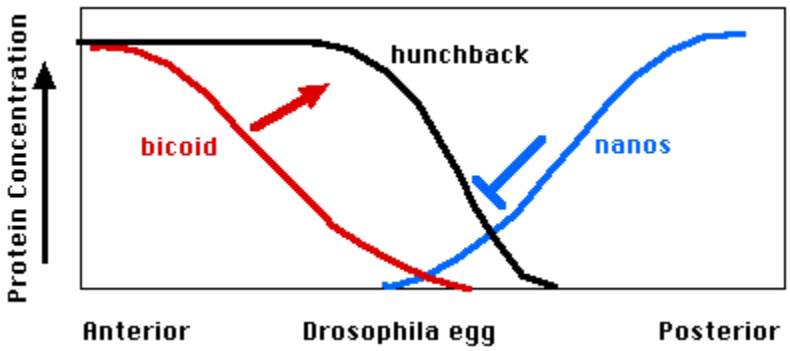


Figure 14.5.1 nanos graph

- **Bicoid** protein is a transcription factor. It binds to the **promoter** of a gene called ***hunchback*** (***hb***), turning it ON (red arrow).
- **Nanos** protein binds to hunchback mRNAs, inhibiting their translation (blue bar).
- These effects combine to produce a high level of hunchback protein at the anterior of the embryo; with a sharp cut-off toward the posterior.
- The hunchback protein is also a transcription factor (as we shall see).
- These concentration gradients regulate the turning on and off of other genes in sharply-defined regions of the embryo.
- These establish the various segments of the body.

Eve stripe 2

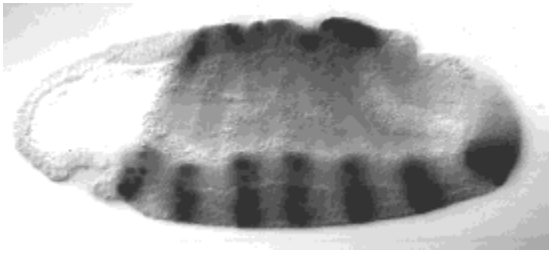


Figure 14.5.2 Gene even skipped

The gene ***even-skipped*** (***eve***) is expressed in 7 bands or stripes corresponding to 7 of Drosophila's 14 segments (skipping the even-numbered ones). The photo (courtesy of Peter A. Lawrence and Blackwell Scientific Publications) shows the 7 stripes of *eve* activation.

At first the gene is expressed in fairly broad zones, but in time its expression becomes restricted to ever-narrower stripes. The mechanism by which this occurs is known for the second stripe.

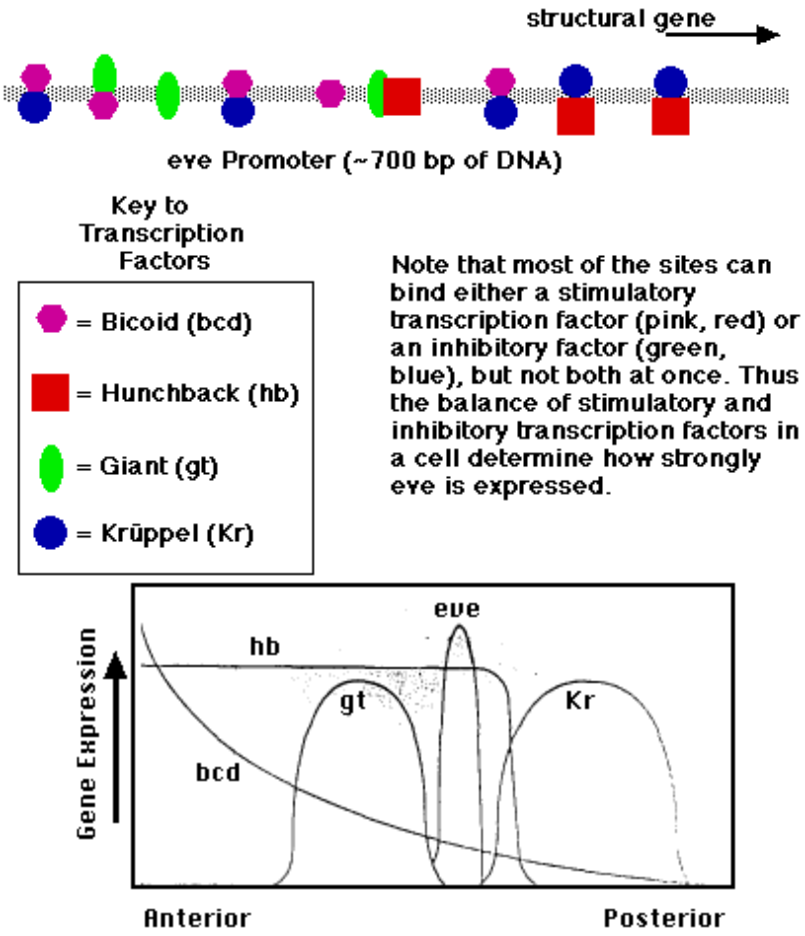


Figure 14.5.4 Eve stripe

The *eve* promoter has binding sites for the proteins encoded by ***bicoid*** (***bcd***), ***hunchback*** (***hb***), ***giant*** (***gt***) and ***Krüppel*** (***Kr***).

- Binding of **bicoid** and **hunchback** proteins **stimulates** transcription of *eve*.
- Binding of **giant** and **Krüppel represses** transcription.

Trapped in a valley between high levels of the giant and Krüppel proteins, expression of *eve* in the second stripe finally becomes limited to a band of cells only one cell thick. (A different set of promoter sites is used in the third eve stripe so expression is not repressed there.)In principle, then, such a system of interacting gradients of transcription factors could act as on-off switches, which in time partition the embryo into its future segments.

Drosophila development (and probably that of animals in general) passes through three rather different (although often overlapping) phases:

- establishing the main axes (dorsal-ventral; anterior-posterior; left-right). This is done by gradients of mRNAs and proteins encoded by the mother's genes and placed in the egg by her.
- establishing the main body parts such as the notochord and central nervous system in vertebrates.and the segments in Drosophila (discussed here). These are run by genes of the zygote itself.
- filling in the details; that is, building the various organs of the animal. (Our example will include the wings, legs, and eyes of Drosophila.)

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14.6: Homeobox Genes

Insect (Drosophila) and frog (Xenopus) development passes through three rather different (although often overlapping) phases: (1) establishing the main axes (dorsal-ventral; anterior-posterior; left-right). This is done by gradients of mRNAs and proteins encoded by the mother's genes and placed in the egg by her. (2) Establishing the main body parts such as the notochord and central nervous system in vertebrates and the segments in Drosophila These are run by genes of the zygote itself.

Now let us look for clues as to how the final working out of the embryo is done. We shall examine four examples:

1. the formation of **wings** (in Drosophila)
2. the formation of **legs** (also in Drosophila)
3. the formation of the **bones** (**radius** and **ulna**) of the front limb in mammals (mice)
4. the formation of **eyes** (probably in all animals)

Wings

The insect body plan consists of head, thorax, and abdomen. The thorax is built from three **segments**, **T1**, **T2**, and **T3**. Each carries a pair of legs; hence insects are six-legged creatures. In most of the insect orders, T2 and T3 each carry a pair of wings (the honeybee is an example). However, **flies** belong to the insect order **diptera**; they have only a single pair of wings (on T2). The third thoracic segment, T3, carries instead a pair of balancing organs called **halteres**.

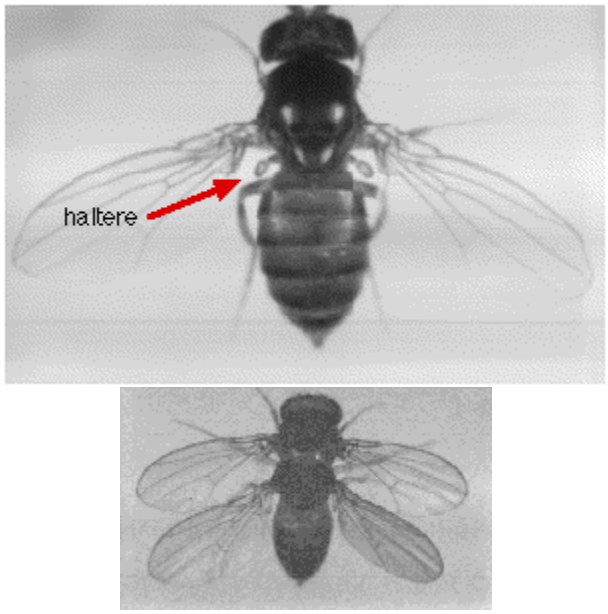


Figure 14.6.1 Flies with haltere Fig.14.6.2 Flies with haltere replaced by second pair of wings

In Drosophila, a gene called ***Ultrabithorax*** (***Ubx***) acts within the cells of T3 to suppress the formation of wings. By creating a double mutation in the *Ultrabithorax* gene (in its introns, as it turned out), Professor E. B. Lewis of Caltech was able to produce flies in which the halteres had been replaced by a **second pair of wings**. *Ultrabithorax* (*Ubx*) is an example of a "**selector gene**". Selector genes are genes that regulate (turning on or off) the expression of other genes. Thus selector genes act as "**master switches**" in development.

Wings and all their associated structures are complicated pieces of machinery. Nonetheless, mutations in a **single gene**, were able to cause the reprogramming of the building of T3 (and deprived the flies of their ability to fly). Selector genes encode **transcription factors**. *Ultrabithorax* encodes a transcription factor that is normally expressed at high levels in T3 (as well as in the first abdominal segment) of Drosophila.

These photographs were taken by, and kindly supplied by, Professor Lewis. He has spent his entire career studying selector genes

in Drosophila. His life's work was honored when he shared the 1995 Nobel Prize for physiology or medicine.

Legs

Another selector gene, called ***Antennapedia*** (***Antp***), is normally turned "on" (expressed) in the thorax and turned "off" (repressed) in the cells of the head. However, mutations in ***Antp*** can cause it to turn on in the head and form a pair of legs where the antennae would normally be.

When you consider the many genes that must be involved in building a complex structure like an insect leg (or wing), it is remarkable that a single gene can switch them all on. It is also clear that once a selector gene turns "on" in certain cells of the embryo, it remains "on" in all the cells derived from those cells. Those cells become irrevocably committed to carrying out the genetic program leading to the formation of a leg or wing.

Homeobox Genes

Most selector genes, including *Antp* and *Ubx*, are homeobox genes

Antp, *Ubx*, and a number of other selector genes have been cloned and sequenced. They all contain within their coding regions a sequence of some 180 nucleotides called a **homeobox**. The approximately 60 amino acids encoded by the homeobox are called a **homeodomain**. It mediates DNA binding by these proteins. Many proteins containing homeodomains have been shown to be transcription factors; probably they all are.

	1-10	11-20	21-30	31-40	41-50	51-60
Antp	RKRGRQTYTRYQTLELEKEFHFNRYLTRRRIEIAHALCLTERQIKIWFQNRRMKWKKEN					
Hox-B7	----------Y----------T----------					
bicoid	PR-T-T-F-SS-IA---OH-LQG----AP-LADLSAK-A-QTA-V----K---RRH-IQS					
goosecoid	KR-H-TIF-DE-LEA--NL-QETK-PDVGT-EQL-RRVH-R-EKVEV--K---A--RRQK					
mab-5	S--T----S-S----------YHK----K--Q--SET-H-----V----------H---A					

Figure 14.6.3: Homeodomains

The table shows the sequence of 60 amino acids in the homeodomain of the protein encoded by the Drosophila homeobox gene ***Antennapedia*** (***Antp***) compared with the homeodomain encoded by the mouse gene ***HoxB7***; by ***bicoid*** (***bcd***), another homeobox gene in Drosophila; by *goosecoid*, a homeobox gene in Xenopus; and by *mab-5*, a homeobox gene in the roundworm *Caenorhabditis elegans*. A dash indicates that the amino acid at that position is identical to the one in the *Antennapedia* homeobox domain. Note that the mouse homeobox in *HoxB7* differs from the *Antp* homeobox by only two amino acids (even though some 700 millions years have passed since these animals shared a common ancestor). *HoxB6*, used in the experiment described in the next section, differs from *Antp* in only 4 amino acids.

The Hox Cluster

Antp and *Ubx* are two of 8 homeobox genes that are linked in a cluster on one Drosophila chromosome. All of them encode transcription factors, each with a DNA-binding homeodomain and act in sequential zones of the embryo in the **same order** that they occur on the chromosome! The entire cluster is designated *HOM-C* with *lab*, *Pb*, *Dfd*, *Scr*, and *Antp* belonging to the *ANT-C* complex and *Ubx*, *Abd-A*, and *Abd-B* designated the *BX-C* complex, All animals that have been examined have at least one *Hox* cluster. Their genes show strong homology to the genes in *Drosophila*. Mice and humans have 4 *Hox* clusters (a total of 39 genes in humans) located on four different chromosomes.

- In mice: *HoxA*, *HoxB* (shown here), *HoxC*, *HoxD*
- In humans: *HOXA*, *HOXB*, *HOXC*, *HOXD*

As in Drosophila, they act along the developing embryo in the same sequence that they occupy on the chromosome. All the genes in the mammalian *Hox* clusters show some sequence homology to each other (especially in their homeobox) but very strong sequence homology to the equivalent genes in Drosophila. *HoxB7* differs from *Antp* at only two amino acids, *HoxB6* at four. In fact, when the **mouse *HoxB6*** gene is inserted in Drosophila, it can substitute for *Antennapedia* and produce legs in place of antennae just as mutant *Antp* genes do. This fascinating result indicates clearly that these selector genes have retained, through millions of years of evolution, their function of assigning particular positions in the embryo, but the structures actually built depend on a

different set of genes specific for a particular species.

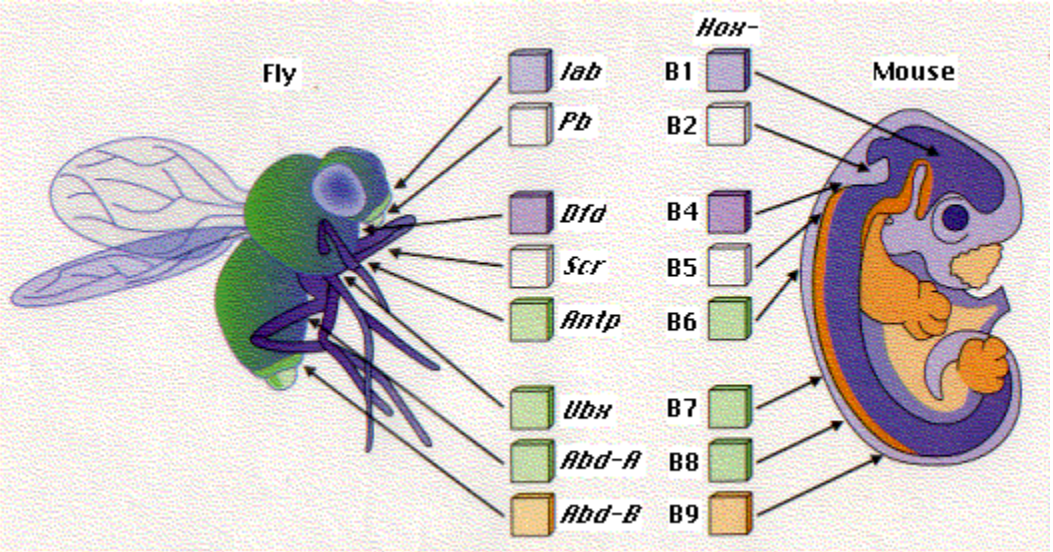


Figure 14.6.4 HOX

The Mammalian Skeleton

The foreleg of the mouse and the arm of humans contain a single upper bone, the humerus, and two lower bones, the radius and ulna. The building of the entire arm, including carpals and the phalanges of the fingers, is controlled by *Hox* cluster genes.

When mice were bred with homozygous mutations for **both *HoxA11* and *HoxD11***, they were born with neither radius nor ulna in the forelimbs. Here, then, is another example of the power of selector genes to initiate a whole program, perhaps involving hundreds of other genes, to form a structure as complex as a forelimb. Mice that are homozygous for mutant *HoxA10*, *C10*, and *D10* genes fail to form a lumbar and sacrum region in their vertebral column ("backbone"). Instead these vertebrae develop ribs like the thoracic vertebrae above them. However, if any one of these 6 *Hox* alleles is normal, the mice are much less severely affected. This shows the high degree of redundancy of these *Hox* genes.

Eyes

The compound eye of Drosophila is a marvel of precisely-organized structural elements. No one knows how many genes it takes to make the eye, but it must be a large number. Nevertheless, a single **selector gene**, ***eyeless*** (***ey***) (named, as is so often the case, for its mutant phenotype) can serve as a master switch turning on the entire cascade of genes needed to build the eye. Through genetic manipulation, it is possible to get the eyeless gene to be expressed in tissues where it is ordinarily not expressed. When ***eyeless*** is turned on in cells destined to form

- the insect's antennae, eyes form on the antennae
- wings, extra eyes form on the wings
- legs, eyes form on the legs.

Mice have a gene, ***small eyes*** (***Sey***; also known as ***Pax6***) that is similar in sequence to the Drosophila *eyeless* gene. As its name suggests, it, too, is involved in eye formation (even though the structure of the mouse eye is entirely different from the compound eye of Drosophila).

However, the sequences of the mouse ***small eyes*** gene and the Drosophila ***eyeless*** genes are so similar that the mouse gene can substitute for eyeless when introduced into Drosophila. So, like the genes of the *Hox* clusters, Drosophila ***eyeless*** and mouse ***small eyes*** have retained, through millions of years of independent evolution, their function of assigning particular positions in the embryo where certain structures should be built, but the structures actually built depend on a different set of genes specific for a particular species.

Humans also have a gene that is homologous to *small eyes* and *eyeless*: it is called ***aniridia***. Those rare humans who inherit a

single mutant version of *aniridia* lack irises in their eyes.

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14.7: Stem Cells

Stem cells are cells that divide by mitosis to form either two stem cells, thus increasing the size of the stem cell "pool", or one daughter that goes on to differentiate, and one daughter that retains its stem-cell properties. How the choice is made is still unknown. However, several genes have been found whose activity prevents a daughter cell from differentiating.

Types of Stem Cells

Several adjectives are used to describe the developmental potential of stem cells; that is, the number of different kinds of differentiated cell that they can become.

1. **Totipotent cells.** In mammals, totipotent cells have the potential to become any type in the adult body and any cell of the extraembryonic membranes (e.g., placenta). The only totipotent cells are the **fertilized egg** and the first 4 or so cells produced by its cleavage (as shown by the ability of mammals to produce identical twins, triplets, etc.). In mammals, the expression totipotent **stem** cells is a misnomer — totipotent cells cannot make more of themselves.
2. **Pluripotent stem cells.** These are true stem cells, with the potential to make any differentiated cell in the body (but probably not those of the placenta which is derived from the trophoblast).

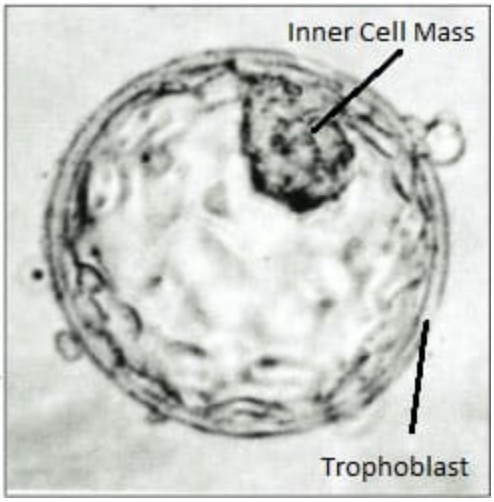


Figure 14.7.1: Human blastocyst showing inner cell mass (top right) and trophoblast. (J. Conaghan).

Three types of pluripotent stem cells occur naturally:

- **Embryonic Stem (ES) Cells.** These can be isolated from the **inner cell mass** (ICM) of the blastocyst — the stage of embryonic development when implantation occurs. For humans, excess embryos produced during in vitro fertilization (IVF) procedures are used. Harvesting ES cells from human blastocysts is controversial because it destroys the embryo, which could have been implanted to produce another baby (but often was simply going to be discarded).
- **Embryonic Germ (EG) Cells.** These can be isolated from the precursor to the gonads in aborted fetuses.
- **Embryonic Carcinoma (EC) Cells.** These can be isolated from teratocarcinomas, a tumor that occasionally occurs in a gonad of a fetus. Unlike the other two, they are usually aneuploid.

All three of these types of pluripotent stem cells can only be isolated from embryonic or fetal tissue. They can be grown in culture, but only with special methods to prevent them from differentiating.

In mice and rats, embryonic stem cells can also:

- contribute to the formation of a healthy chimeric adult when injected into a blastocyst which is then implanted in a surrogate mother;
- enter the germline of these animals; that is, contribute to their pool of gametes;
- develop into teratomas when injected into immunodeficient (SCID) mice. These tumors produce a wide variety of cell types

representing all three germ layers (ectoderm, mesoderm, and endoderm).

Using genetic manipulation in the laboratory, pluripotent stem cells can now be generated from differentiated cells. These **induced pluripotent stem cells** (iPSCs) are described below.

3. **Multipotent stem cells.** These are true stem cells but can only differentiate into a limited number of types. For example, the bone marrow contains multipotent stem cells that give rise to all the cells of the blood but not to other types of cells. Multipotent stem cells are found in adult animals; perhaps most organs in the body (e.g., brain, liver, lungs) contain them where they can replace dead or damaged cells. These **adult stem cells** may also be the cells that — when one accumulates sufficient mutations — produce a clone of cancer cells.

Stem Cells for Human Therapy

The Dream

Many medical problems arise from damage to differentiated cells. Examples:

- Type 1 diabetes mellitus where the beta cells of the pancreas have been destroyed by an autoimmune attack
- **Parkinson's disease**; where dopamine-secreting cells of the brain have been destroyed
- Spinal cord injuries leading to paralysis of the skeletal muscles
- Ischemic stroke where a blood clot in the brain has caused neurons to die from oxygen starvation
- **Multiple sclerosis** with its loss of myelin sheaths around axons
- Blindness caused by damage to the cornea

The great developmental potential of stem cells has created intense research into enlisting them to aid in replacing the lost cells of such disorders. While progress has been slow, some procedures already show promise. Using multipotent "adult" stem cells.

- culturing human epithelial stem cells and using their differentiated progeny to replace a damaged cornea. This works best when the stem cells are from the patient (e.g. from the other eye). Corneal cells from another person (an allograft) are always at risk of rejection by the recipient's immune system.
- the successful repair of a damaged left bronchus using a section of a donated trachea that was first cleansed of all donor cells and then seeded with the recipient's epithelial cells and cartilage-forming cells grown from stem cells in her bone marrow. So far the patient is doing well and needs no drugs to suppress her immune system.

Using differentiated cells derived from embryonic stem (ES) cells. Phase I clinical trials are underway to assess the safety of

- injecting retinal cells derived from ES cells
  - into the eyes of young people with an inherited form of juvenile blindness;
  - into the eyes of adults with age-related macular degeneration.
- injecting glial cells derived from ES cells into patients paralyzed by spinal cord injuries.

The Immunological Problems

One major problem that must be solved before human stem cell therapy becomes a reality is the threat of rejection of the transplanted cells by the host's immune system (if the stem cells are allografts; that is, come from a genetically-different individual).

A Possible Solution

One way to avoid the problem of rejection is to use stem cells that are genetically identical to the host. This is already possible in the rare situations when the patient has healthy stem cells in an undamaged part of the body (like the stem cells being used to replace damaged corneas). But even where no "autologous" stems cells are available, there may be a solution: using **somatic-cell nuclear transfer** .

In this technique,

1. An egg has its own nucleus removed and replaced by
2. a nucleus taken from a somatic (e.g., skin) cell of the donor.
3. The now-diploid egg is allowed to develop in culture to the blastocyst stage when
4. embryonic stem cells can be harvested and grown up in culture.
5. When they have acquired the desired properties, they can be implanted in the donor with no fear of rejection.

Using this procedure it possible to not only grow blastocysts but even have these go on to develop into adult animals — **cloning** — with a nuclear genome identical to that of the donor of the nucleus. The first successful cloning by SCNT was with amphibians. Later, mammals such as sheep (Dolly), cows, mice and others were successfully cloned. And in the 11 November 2007 issue of **Science**, researchers in Oregon reported success with steps 1–4 in rhesus monkeys (primates like us).

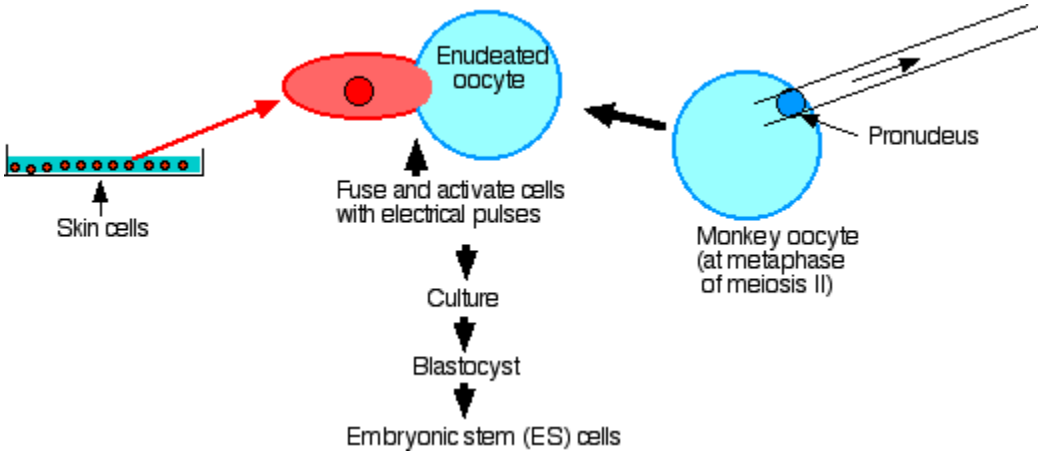


Figure 14.7.2: Somatic cell transfer in rhesus monkeys

Their procedure:

- Remove the spindle and thus all nuclear material from secondary oocytes at metaphase of meiosis II.
- Fuse each enucleated egg with a skin cell taken from a male monkey.
- Culture until the blastocyst stage is reached.
- Extract embryonic stem cells from the inner cell mass.
- Establish that they have the nuclear genome of the male (but mostly the mitochondrial genome of the female).
- Culture with factors to encourage differentiation: they grew cardiac muscle cells (which contracted), and even neuron-like cells.
- Inject into SCID mice and examine the tumors that formed. These contained cells of all three germ layers: ectoderm, mesoderm, and endoderm.
- However, even after more than 100 attempts, they have not been able to implant their monkey blastocysts in the uterus of a surrogate mother to produce a cloned monkey.

This should reassure people who view with alarm the report in May 2013 by the same workers that they have finally succeeded in producing embryonic stem cells (ESCs) using SCNT from differentiated human tissue. The workers assure us that they will not attempt to implant these blastocysts in a surrogate mother to produce a cloned human. And their failure with monkeys suggests that they would fail even if they did try.

While cloning humans still seems impossible, patient-specific ESCs

- could be used in cell-replacement therapy or, failing that,
- provide the material for laboratory study of the basis of — and perhaps treatment of — genetic diseases.

Whether they will be more efficient and more useful than induced pluripotent stem cells remains to be seen.

Questions that Remain to be Answered

- **Imprinted Genes.** Sperm and eggs each contain certain genes that carry an "imprint" identifying them later in the fertilized egg as being derived from the father or mother respectively. Creating an egg with a nucleus taken from an adult cell may not

allow a proper pattern of imprinting to be established. When the diploid adult nucleus is inserted into the enucleated egg (at least those of sheep and mice), the new nucleus becomes "reprogrammed". What reprogramming actually means still must be learned, but perhaps it involves the proper methylation and demethylation of imprinted genes. For example, the inactive X chromosome in adult female cells must be reactivated in the egg, and this actually seems to happen.

- **Aneuploidy.** In primates (in contrast to sheep, cattle, and mice), the process of removing the resident nucleus causes molecules associated with the centrosome to be lost as well. Although injecting a donor nucleus allows mitosis to begin, spindle formation may be disrupted, and the resulting cells fail to get the correct complement of chromosomes (aneuploidy).
- **Somatic Mutations.** This procedure also raises the spectre of amplifying the effect(s) of somatic mutations. In other words, mutations that might be well-tolerated in a single somatic cell of the adult (used to provide the nucleus) might well turn out to be quite harmful when they become replicated in a clone of cells injected later into the patient.
- **Political Controversy.** The goal of this procedure (which is often called **therapeutic cloning** even though no new individual is produced) is to culture a blastocyst that can serve as a source of **ES cells**. But that same blastocyst could theoretically be implanted in a human uterus and develop into a baby that was genetically identical to the donor of the nucleus. In this way, a human would be cloned. And in fact, Dolly and other animals are now routinely cloned this way. The spectre of this is so abhorrent to many that they would like to see the procedure banned despite its promise for helping humans. In fact, many are so strongly opposed to using human blastocysts — even when produced by nuclear transfer — that they would like to limit stem cell research to adult stem cells (even though these are only multipotent).

Possible Solutions to the Ethical Controversy

Induced pluripotent stem cells (iPSCs)

A promising alternative to the use of embryonic stem cells in human therapy are recently-developed methods of genetically reprogramming the nuclei of differentiated **adult** cells so that they regain the pluripotency of embryonic stem (ES) cells.

In June 2007, three laboratories reported that introducing extra copies of only 4 genes into adult mouse skin cells (fibroblasts) enables them to regain the properties of ES cells. When these cells, named **induced pluripotent stem cells** (iPSCs for short), were placed in mouse blastocysts, they participated in building all the tissues of the chimeric mice that resulted. (When placed in tetraploid (**4n**) blastocysts — unable by themselves to develop normally — embryos were formed that thus were clones of the skin cell donor.) The four genes: *c-Myc*, *Sox2*, *Oct3/4*, *Klf4*.

By 2009, several labs had succeeded in producing fertile adult mice from iPSCs derived from mouse embryonic fibroblasts. This shows that iPSCs are just a capable of driving complete development (pluripotency) as embryonic stem cells.

Reprogramming works in humans, too! Using the same four genes, the Yamanaka lab in Japan reported on 20 November 2007, that they now had reprogrammed human skin cells to become induced pluripotent stem cells (**iPSCs**). And the Thomson lab in Wisconsin accomplished the same thing using *SOX2*, *OCT4*, *NANOG*, and *LIN28*.

Further evidence of the remarkable role played by these few genes is the finding that during normal embryonic development of the zebrafish, the same or similar genes (SoxB1, Oct4, Nanog) are responsible for turning on the genes of the zygote. Earlier in development of the blastula, all the genes being expressed (including these) are the mother's — mRNAs and proteins that the mother deposited in the unfertilized egg. It makes sense that the same proteins that can reprogram a differentiated cell into a pluripotent state (iPSCs) are those that produce the pluripotent cells of the early embryo.

These achievements open the possibility of

- creating cells for laboratory study of the basis of genetic diseases.
  Examples: researchers have succeeded in deriving iPSCs from
  - patients with amyotrophic lateral sclerosis (ALS, "Lou Gehrig's disease"), and then causing them to differentiate into motor neurons (the cells affected in the disease) for study of their properties;
  - the skin cells of a patient with an inherited heart disease (long QT syndrome) and causing these to differentiate into beating heart cells for study in the laboratory.
  - The Jaenisch lab reported in the 6 March 2009 issue of **Cell** that they have succeeded in making iPSCs (they call them hiPSCs) from fibroblasts taken from patients with Parkinson's disease. The cells were then differentiated into dopamine-releasing cells — the cells lacking in this disease. What is particularly exciting is that they accomplished this after using the Cre-lox system to remove all the genes (e.g., *SOX2*, *OCT4*, *KLF4*) needed for reprogramming the fibroblasts to an embryonic-stem-cell-like condition.

    - Since that report, other laboratories — using other methods — have also created iPSCs from which all foreign DNA (vector and transgenes) has been removed. Not only should such cells be safer to use in therapy, but these results show that the stimulus to reprogram a differentiated cell into a pluripotent state need only be transitory.
- creating patient-specific cell transplants — avoiding the threat of immunological rejection — that could be used for human therapy.

  Therapy with iPSCs has already been demonstrated in mice. Three examples:

  1. The Jaenisch lab in Cambridge, MA reported (in **Science**, 21 December 2007) that they had successfully treated knock-in mice that make sickle-cell hemoglobin with the human β^S genes (and show many of the signs of sickle-cell disease in humans) by
    - harvesting some fully-differentiated fibroblasts from a sickle-cell mouse;
    - reprogramming these to become iPSCs by infecting them with *Oct4*, *Sox2*, *Klf4*, and *c-Myc*;
    - then removing (using the Cre-lox system) the *c-Myc* to avoid the danger of this oncogene later causing cancer in the recipient mice;
    - replacing the β^S genes in the iPSCs with normal human β^A genes;
    - coaxing, with a cocktail of cytokines, these iPSCs to differentiate in vitro into hematopoietic (blood cell) precursors;
    - injecting these into sickle-cell mice that had been irradiated to destroy their own bone marrow (as is done with human bone marrow transplants). (Although the recipient mice were different animals from the fibroblast donor, they were of the same inbred strain and thus genetically the same — like identical human twins. So the procedure fully qualifies as "**patient-specific**", i.e., with no danger of the injected cells being rejected by the recipient's immune system.)

  The result: all the signs of sickle-cell disease (e.g., anemia) in the treated animals showed marked improvement.

  2. In the 25 July 2013 issue of *Nature*, a team of Japanese scientists report that they were able to manufacture three-dimensional buds of human liver cells. Their process:
    - create human iPSCs from human fibroblasts using the techniques described above;
    - treat these with the substances needed for them to differentiate in liver cell precursors;
    - culture these with a mixture of human endothelial cells and mesenchymal stem cells (to mimic the conditions that occur in normal embryonic development of the liver);
    - implant the resulting solid masses (buds) of liver-like cells into immunodeficient mice.

  The result: the implanted buds developed a blood supply and the mice began to secrete human albumin, human alpha-1-antitrypsin, and to to detoxify injected chemicals just as human livers do.

  3. Workers in the Melton lab at Harvard University reported in the 9 October 2014 issue of **Cell** that they had succeeded in differentiating large numbers of human beta cells from human iPSCs (as well as from human ES cells). When transplanted into diabetic mice, these cells brought their elevated blood sugar levels back down.

Let us hope that what works in mice can someday be developed into a safe therapy that will work in humans. (In the case of Type 1 diabetes mellitus, however, even patient-derived beta cells will still be at risk of the same autoimmune rejection that caused the disease in the first place.)

Despite these successes, iPSCs may not be able to completely replace the need for embryonic stem cells and may even be dangerous to use in human therapy. Several groups have found that human iPSCs contain mutations as well as epigenetic patterns (e.g., methylation of their DNA) that are not found in embryonic stem cells. Some of the mutations are also commonly found in cancer cells.

Other approaches being explored

- ES cells can be derived from a **single cell** removed from an 8-cell **morula**. The success of preimplantation genetic diagnosis (PGD) in humans shows that removing a single cell from the morula does not destroy it — the remaining cells can develop into a blastocyst, implant, and develop into a healthy baby. Furthermore, the single cell removed for PGD can first be allowed to divide with one daughter used for PGD and the other a potential source of an ES cell line.

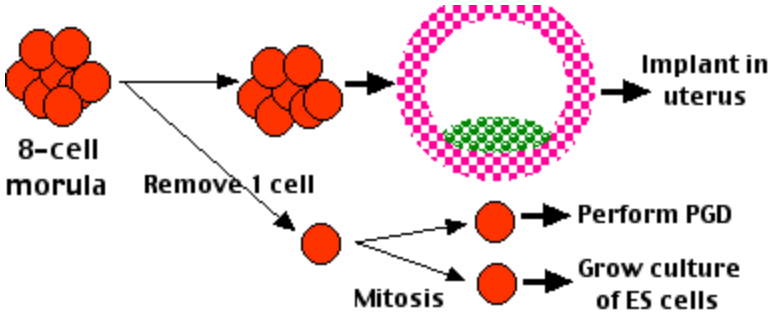


Figure 14.7.1: Preimplantation using ES cell

- In altered nuclear transfer (**ANT**) — a modified version of SCNT (somatic-cell nuclear transfer) — a gene necessary for later implantation (*Cdx2* — encoding a homeobox transcription factor) is turned off (by RNA interference) in the donor nucleus before the nucleus is inserted into the egg. The blastocyst that develops
  - has a defective trophoblast that cannot implant in a uterus
  - but the cells of the inner cell mass are still capable of developing into cultures of ES cells. (The gene encoding the interfering RNA can then be removed using the Cre/*loxP* technique.)
- Jose Cibelli and his team at Advanced Cell Technology reported in the 1 February 2002 issue of **Science** that they had succeeded inIf this form of cloning by parthenogenesis works in humans [It does! — success with unfertilized human eggs was reported in June 2007.], it would have
  - stimulating monkey oocytes to begin dividing without completing meiosis II (therefore still **2n**)
  - growing these until the blastocyst stage, from which they were able to harvest
  - ES cells.
  - the **advantage** that no babies could be produced if the blastocyst should be implanted (two identical genomes cannot produce a viable mammal — probably because of incorrect imprinting);
  - the **disadvantage** that it will only help females (because only they can provide an oocyte!) (But men may have a procedure that works for them next.)
- On 24 March 2006, **Nature** published an online report that a group of German scientists had been able to derive pluripotent stem cells from the stem cells that make spermatogonia in the mouse. Both in vitro and when injected into mouse blastocysts, these cells differentiated in a variety of ways including representatives of all three germ layers. If this could work in humans, it would
  - provide a source of stem cells whose descendants would be "patient-specific"; that is, could be transplanted back into the donor (men only!) without fear of immune rejection.
  - avoid the controversy surrounding the need to destroy human blastocysts to provide embryonic stem cells.
- The 7 January 2007 issue of **Nature Biotechnology** reports the successful production of amniotic fluid-derived stem cells ("AFS"). These are present in the amniotic fluid removed during amniocentesis. With the proper culture conditions, they have been shown to be able to differentiate into a variety of cell types includingSo these cells are pluripotent. Although perhaps not as versatile as embryonic stem cells, they are more versatile than adult stem cells.
  - ectoderm (neural tissue)
  - mesoderm (e.g., bone, muscle)
  - endoderm (e.g., liver)

Applied to humans, none of the above procedures would involve the destruction of a potential human life.

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14.8: Embryonic Stem Cells

The other pages describe:

- the properties and potential therapeutic applications of embryonic (and other types of) stem cells
- how mouse embryonic stem cells can be used to make transgenic mice
- how the fusion of a differentiated cell from an adult sheep with an enucleated sheep egg can produce a **clone** of the cell donor ("Dolly")

The techniques used in the **early steps** of each process have been achieved with human cells.

Thirteen years ago a research team led by James Thomson of the University of Wisconsin reported (in the 6 November 1998 issue of **Science**) that they were able to grow human embryonic stem (**ES**) cells in culture.

At the time of implantation, the mammalian embryo is a **blastocyst**. It consists of the

- **trophoblast** — a hollow sphere of cells that will go on to implant in the uterus and develop into the placenta and umbilical cord.
- **inner cell mass** (**ICM**) that will develop into the baby as well as the extraembryonic amnion and yolk sac.

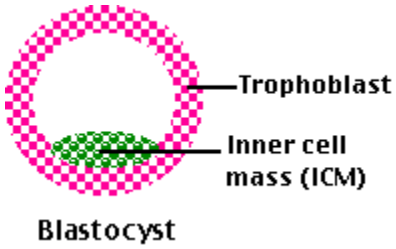


Figure 14.8.1 Blastocyst

The cells of the inner cell mass are considered **pluripotent**; that is, each is capable of producing descendants representing all of the hundreds of differentiated cell types in the newborn baby, including

- ectodermal cells like neurons and skin (epithelial cells)
- mesodermal cells like striated muscle, smooth muscle, cartilage, and bone
- endodermal cells like the liver and the lining of the intestine

The Process

- Remove the trophoblast cells from a human blastocyst (these were extras not needed for assisted reproductive technology).
- Separate the cells of the **inner cell mass** and culture them on a plate of "feeder" cells (mouse fibroblasts were used).
- Isolate single cells and grow them as clones.
- Test the clones.

The Results

- Each successful clone maintained a normal human karyotype (unlike most cultured human cells — HeLa cells, for example).
- These cells had high levels of the enzyme **telomerase**, which maintains normal chromosome length and is characteristic of cells with unlimited potential to divide ("immortal").
- When injected into **SCID mice**, these cells formed **teratomas**; tumors containing a mix of **differentiated** human cell types, including cells characteristic of
  - ectoderm
  - **mesoderm**

- **endoderm**

Note

SCID = **severe combined immunodeficiency**.
SCID mice lack a functioning immune system (have neither T cells nor B cells) and so cannot reject foreign tissue. Some rare inherited diseases of humans are also called SCID. They produce a similar phenotype but involve different molecular defects.

Human embryonic stem cells have the potential to

- teach us about the process of human embryonic development, its genetic control, etc.
- provide a source of replacement cells to repair damaged human tissue. As the proper signals are discovered, it will be possible to cause these cells to differentiate along a **particular pathway**, e.g., to form insulin-secreting beta cells of the islets of Langerhans. Such cells might be able to replace lost or non-functioning cells in a human patient (e.g., with Type 1 diabetes mellitus).

However, there are problems that remain to be solved before this hope can be realized.

- Production of human ES cells requires the destruction of the blastocyst, and this is morally-repugnant to many people.
- Cell replacement therapy had better be "patient-specific"; that is, the donated cells should be genetically identical to the recipient. Otherwise, the replaced cells are at risk of being rejected by the host's immune system. [Link to a discussion of "therapeutic cloning" — a method to avoid this.
- ES cells are pluripotent and might differentiate in unwanted ways when introduced into the patient.

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14.9: Germline vs. Soma

Could a mutation in one of your liver cells ever be passed on to your children?

No!

Why not?

- The fusion of one sperm cell and one egg cell represents the only genetic link between the bodies of parents and the body of their child and
- the cells destined to produce sperm and eggs are set aside very early in embryonic life.

Example 1:

By the 15th week of gestation, the human female fetus has already set aside each and every cell that may someday develop into a mature egg. (In fact, each of these cells has already entered its final meiotic division!)

Example 2:

Caenorhabditis elegans is a microscopic (~ 1 mm) nematode (roundworm) that normally lives in soil. Like all animals, it starts life as a fertilized egg (zygote) which then undergoes the mitotic divisions needed to produce the

- 556 cells of the newly-hatched worm and, later,
- the 959 somatic cells, and a variable number of germ cells, of the adult worm.

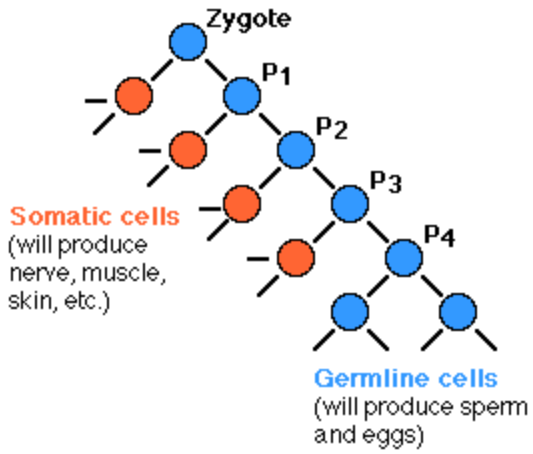


Figure 14.9.2 Germplasts

In Weismann's view, the somaplasm simply provides the housing for the germplasm, seeing to it that the germplasm is protected, nourished, and conveyed to the germplasm of the opposite sex to create the next generation. The old riddle about which came first, the chicken or the egg, would have been no puzzle to Weismann. In his view, the chicken is simply one egg's device for laying another egg.

Weismann also understood the implications of his theory for aging. Once the opportunity to pass germplasm on has passed, there is no need to maintain the integrity of the somaplasm ("disposable soma"); hence the decline in body function with aging .

Today we know that only the germplasm — the gametes and the cells that form them — continue to express high levels of the enzyme telomerase. These cells are able to maintain the length of their chromosomes forever and are immortal. The cells of the somaplasm, in contrast, stop producing telomerase, lose a portion of their chromosome tips at each mitosis, and eventually die.

Deciding between germline and soma

What determines

- which of the early cells of the embryo will be destined to go on to form sperm or eggs, that is to become **germline** and
- which are destined to develop into the body tissues (**soma**) of the animal?

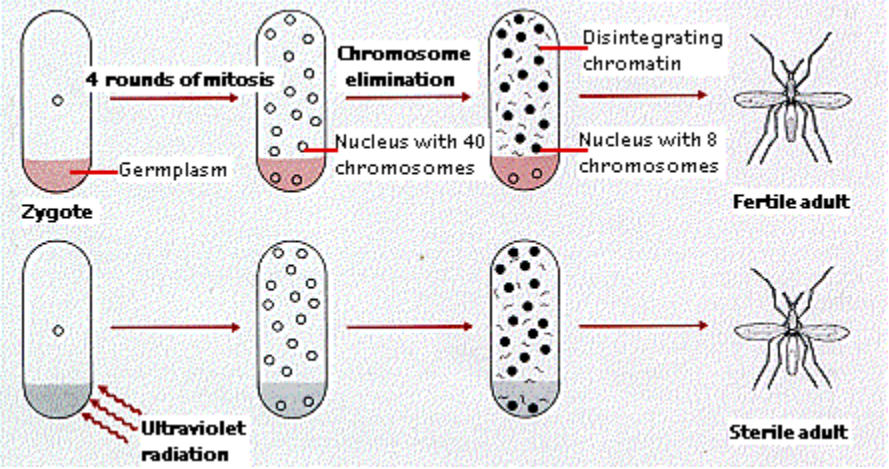


Figure 14.9.3 Mitotic division in a gall midge

At the 4th mitotic division in the gall midge (an insect) egg, 2 of the 16 nuclei become pinched off in a small amount of cytoplasm at one end of the egg. At the 5th mitosis, these two nuclei divide normally, producing daughter cells with the full complement of chromosomes (2**n** = 40) of the species. But not so for the other nuclei. When each of these reaches anaphase, only 8 of their 40 chromosomes (dyads) separate and move to opposite ends of the spindle. The remaining 32 chromosomes stay at the metaphase plate and eventually disintegrate.

- The descendants of the two "normal" nuclei ultimately differentiate to form sperm or eggs, i.e., the germline.
- The descendants of the rest of the nuclei, those with the sharply-reduced chromosome number, go on to form all the other tissues of the insect body, i.e., the soma.

The top row of this figure shows normal development in the gall midge. The gametes are descended from the two nuclei, each with a full diploid set of 40 chromosomes, that were partitioned off in a mass of special cytoplasm (here called "germplasm"). The remaining nuclei lose 32 chromosomes before going on to form the rest of the insect body (the somaplasm).

The bottom row shows that destruction of the germplasm causes the nuclei that move there to undergo chromosome elimination also. The animal that develops is sterile but otherwise normal.

Mammals

In mammals (and birds), the decision to become germ cells is **not** intrinsic but is the result of **cell-to-cell signaling** during early embryonic development.

In mice, the setting aside of the future germ cells begins at the start of gastrulation (6 days after fertilization in the mouse) as the mesoderm is forming. **Ecto**derm cells of the future extraembryonic membranes secrete cytokines (including bone morphogenic proteins) which signal cells in the developing **meso**derm to differentiate into cells which — under the influence of other inter-cellular chemical signals — will go on to form either

- mesoderm of the extraembryonic membranes (amnion and allantois)
- primordial germ cells (**PGC**s)

The PGCs migrate into the part of the developing embryo that will go on to form the gonads (**ovaries** or **testes**).

Exceptions to Weismann's Theory

The distinction between germline and soma exists **only in animals**.

In **plants**, cells destined to become gametes do arise from somatic tissues. In the flowering plants (angiosperms), for example, certain signals cause meristems that had been making stem tissue to become converted into flower buds which go on to make the gametes.

In **microorganisms**, all life's functions are embodied in a single cell. (However, some unicellular organisms, like the ciliated protozoan **Tetrahymena thermophila**, have a complete genome in their **micronuclei**, which are passed on to the next generation, as well as genes in a **macronucleus**, which is not. Thus, even here, there is the equivalent of a distinction between germline and soma.)

Somatic vs. Germline Mutations

The significance of mutations is profoundly influenced by the distinction between germline and soma. Mutations that occur in a **somatic cell**, in the bone marrow or liver for example, may

- damage the cell
- make the cell cancerous
- kill the cell

Whatever the effect, the ultimate fate of that **somatic mutation** is to disappear when the cell in which it occurred, or its owner, dies.

Germline mutations, in contrast, will be found in every cell descended from the zygote to which that mutant gamete contributed. If an adult is successfully produced, every one of its cells will contain the mutation. Included among these will be the next generation of gametes, so if the owner is able to become a parent, that mutation will pass down to yet another generation.

Example 1:

More than 8000 people living in South Africa today carry a gene for the metabolic disease called **porphyria**. Every one of them has acquired their gene through a chain of ancestors leading back to a single couple: Ariaantje Jacobs and Gerrit Jansz. This woman and man emigrated from Holland to South Africa late in the seventeenth century and one or the other of them passed the gene — **through the germline** — on to their descendants. Fortunately, the ailment is usually mild (unless the person is given a barbiturate sedative, which triggers a violent reaction).

Example 2:

Retinoblastoma, a tumor that occurs in humans in

- a **sporadic** form, which is caused by a **somatic mutation** in each of the **two *RB* genes** in a cell
- a **familial** (inherited) form, which is caused by a somatic mutation to one *RB* gene in a cell that already has one mutant *RB* gene inherited through the germline from a parent.

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14.10: Regeneration

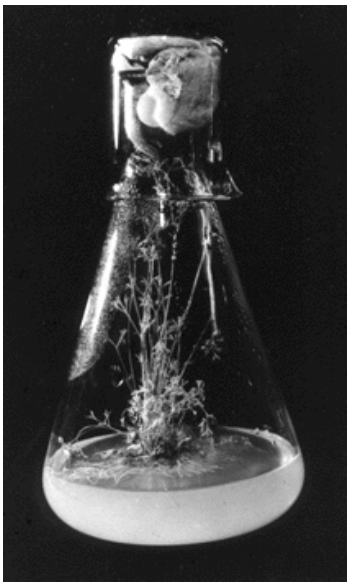


Figure 14.10.2 Dugesia and Dugesia regenaration

When some species of flatworms (left) are decapitated, they can regenerate a new head. Double-amputees can regenerate both a new head at the anterior surface and a new tail at the posterior surface (right). They do this by the proliferation and differentiation of the pluripotent stem cells (called neoblasts) that it retains in its body throughout its life.

How do the cells know whether to develop into a head or a tail? Thanks to the ease with which individual genes can be knocked out by RNA interference (RNAi), it has been shown that Wnt/β-catenin signaling dictates where the head and tail form.

- Blocking Wnt/β-catenin signaling by RNAi causes a head to form where a tail should (producing a two-headed animal) while
- blocking part of the β-catenin degradation complex (thus enhancing the pathway) causes a tail to develop where a head should (producing a two-tailed animal).

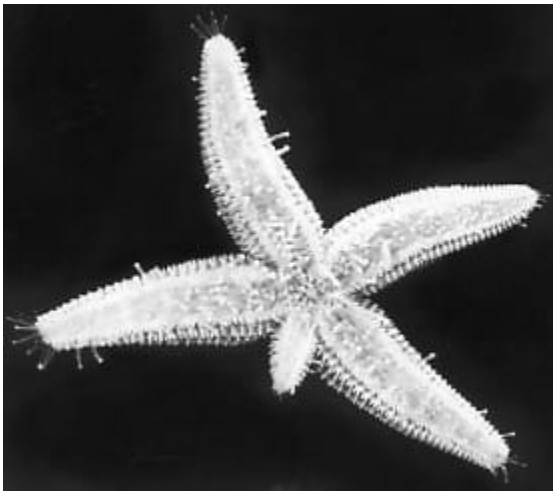


Figure 14.10.4 Salamander

These amphibians can regenerate a missing tail, legs, even eyes. This remarkable ability is particularly pronounced in the larval stage. For this reason, larval salamanders are favorites for doing research on regeneration. For example, cutting the tail off a larval salamander initiates the following sequence of events:

- A layer of epidermal cells grows over and covers the stump.
- A mass of undifferentiated cells — called the **blastema** — develops just beneath.
- Muscle and cartilage form in the regrowing tail.
- The notochord and spinal cord grow out into the regrowing tail.
- After a few weeks, a new, fully-functional and anatomically-correct tail is complete.

The Mechanism

For years, it has been unclear as to whether this regeneration depends on

- a population of pluripotent stem cells that have resided in the animal body prepared for such an event (as occurs in the hydra) or
- the **dedifferentiation** of specialized cells, e.g. muscle and cartilage cells, in the stump.

The answer appears to be both.

- Stem cells in the spinal cord migrate into the regrowing tail and differentiate into several cell types, including muscle and cartilage. Although the stem cells are ectoderm, they are able to develop into mesoderm.
- Muscle cells in the stump migrate into the blastema while
  - reentering the cell cycle to produce thousands of descendants;
  - **dedifferentiate** as they do so; that is, they lose the characteristic proteins, etc. of muscle cells.
- Even though there is as yet no sign of a tail, its final pattern is established during this process for if the blastema is removed and transplanted elsewhere, it will continue the process of regenerating a tail.
- Finally the cells of the blastema differentiate into all the cell types — nerve, muscle, cartilage, skin — used to build the regenerated tail.

Mammals

Don't we wish that we had the same powers of regeneration that salamanders do: able to regenerate a severed spinal cord or grow a new heart! But unfortunately, we cannot. We can regenerate some skin, a large amount of liver, and the very tips of fingers and toes. But that's about it. Just why we are so limited is not known (but is the subject of intense research). Much of the excitement surrounding research on stem cells is because of the hope that they may provide a means of regrowing damaged or lost tissues or even organs.

In contrast to the situation that appears to hold for salamanders, dedifferention of specialized cells does not appear to play a role in the formation of a blastema in mice. Instead, the various tissues — epidermis, hair follicles, sweat glands, neurons (all ectoderm) and muscle, bone, tendon, blood vessels (mesoderm) — that participate in regenerating the tip of an amputated mouse digit (finger or toe) develop from a diverse population of "adult" stem cells in the stump that retain their restricted developmental potential. You can read about the evidence for this in Rinkevich, Y., *et al.*, *Nature*, **476**, 409-413 (25 August 2011).

Genetic Control of Regeneration

A number of genes have been found to be implicated in regeneration. One of the most potent of these is *Wnt*.

- Injection of agents (e.g. antisense RNA molecules) that interfere with the Wnt/β-catenin pathway
  - blocks limb regeneration in salamanders and, as we saw above,
  - promotes head formation in regenerating planarians, while
- injection of agents that enhance the Wnt/β-catenin pathway
  - enable chicks (that, like mammals, are normally incapable of regenerating limbs) to regenerate a wing;
  - as well as enabling a regenerating planarian to form a tail where a head should go.

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UNIT 15: THE ANATOMY AND PHYSIOLOGY OF ANIMALS

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15.1C: Nutrition

15.1D: Recommended Dietary Allowances

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15.1: NUTRITION



- Topic hierarchy

15.1A: The Human Gastrointestinal Tract

15.1B: Metabolism

15.1C: Nutrition

15.1D: Recommended Dietary Allowances

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15.1A: The Human Gastrointestinal Tract

Strategy and Topology

Humans (and most animals) digest all their food extracellularly; that is, outside of cells. Digestive enzymes are secreted from cells lining the inner surfaces of various **exocrine glands**. The enzymes hydrolyze the macromolecules in food into small, soluble molecules that can be absorbed into cells.

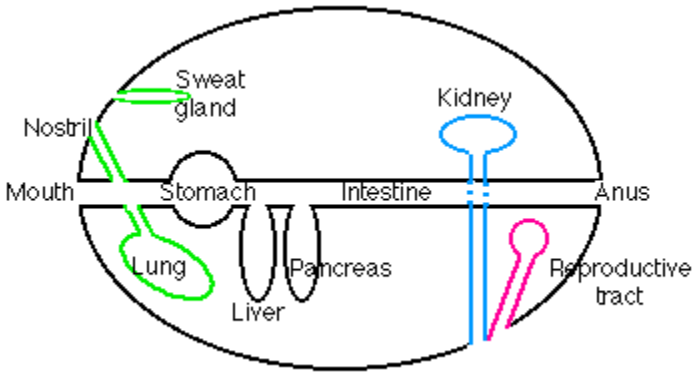


Figure 15.1.1.1: Human Topology

Figure 15.1.1.1 shows the major topological relationships in the body. The linings of all

- exocrine glands, including digestive glands
- nasal passages, trachea, and lungs
- kidney tubules, collecting ducts, and bladder
- reproductive structures like the vagina, uterus, and fallopian tubes

are all continuous with the surface of the body. Anything placed within their lumen is, strictly speaking, outside the body. This includes the secretions of all **exocrine glands** (in contrast to the secretions of endocrine glands, which are deposited in the blood) and any indigestible material placed in the mouth which will appear, in due course, at the other end.

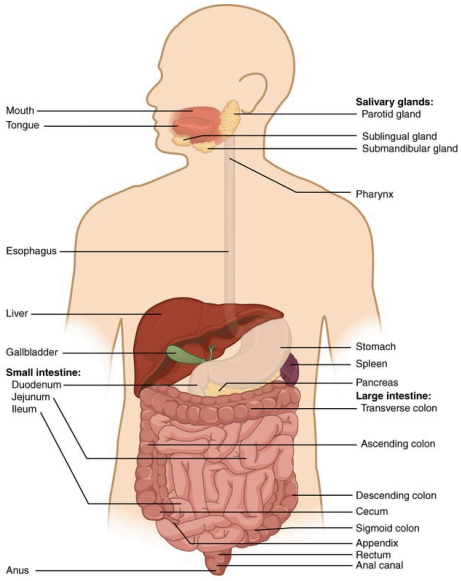


Figure 15.1A.1: Components of the Digestive System. All digestive organs play integral roles in the life-sustaining process of digestion. (CC BY 3.0; OpenStax).

Ingestion

Food placed in the mouth is ground into finer particles by the teeth. It is then moistened and lubricated by saliva (secreted by three pairs of **salivary glands**). Small amounts of starch are digested by the **amylase** present in saliva. The resulting bolus of food is swallowed into the **esophagus** and carried by **peristalsis** to the stomach.

The Stomach

The wall of the stomach is lined with millions of **gastric glands**, which together secrete 400–800 ml of gastric juice at each meal. Several kinds of cells are found in the gastric glands inlcuding parietal cells, chief cells, mucus-secreting cells, and hormone-secreting (endocrine) cells.

Parietal cells

Parietal cells secrete **hydrochloric acid** and **intrinsic factor**.

Hydrochloric acid (HCl)

Parietal cells contain a **H^+/K^+ ATPase**. This transmembrane protein secretes H^+ ions (protons) by active transport, using the energy of ATP. The concentration of H^+ in the gastric juice can be as high as 0.15 M, giving gastric juice a pH somewhat less than 1. With a concentration of H^+ **within** these cells of only about 4×10^{-8} M, this example of active transport produces more than a million-fold increase in concentration. No wonder that these cells are stuffed with mitochondria and are extravagant consumers of energy.

Intrinsic factor is a protein that binds ingested vitamin B_{12} and enables it to be absorbed by the intestine. A deficiency of intrinsic factor — as a result of an autoimmune attack against parietal cells — causes **pernicious anemia**.

Chief cells

The chief cells synthesize and secrete **pepsinogen**, the precursor to the proteolytic enzyme **pepsin**. Pepsin cleaves peptide bonds, favoring those on the C-terminal side of tyrosine, phenylalanine, and tryptophan residues. Its action breaks long polypeptide chains into shorter lengths. Secretion by the gastric glands is stimulated by the hormone gastrin. Gastrin is released by endocrine cells in the stomach in response to the arrival of food.

Absorption in the stomach

Very little occurs. However, some water, certain ions, and such drugs as aspirin and ethanol are absorbed from the stomach into the blood (accounting for the quick relief of a headache after swallowing aspirin and the rapid appearance of ethanol in the blood after drinking alcohol). As the contents of the stomach become thoroughly liquefied, they pass into the **duodenum**, the first segment (about 10 inches [25 cm] long) of the small intestine. Most of our ingested vitamins and minerals are absorbed here. Two ducts enter the duodenum:

1. one draining the gall bladder and hence the **liver** and
2. the other draining the exocrine portion of the **pancreas.**

The Liver

The liver secretes **bile**. Between meals it accumulates in the gall bladder. When food, especially when it contains fat, enters the duodenum, the release of the hormone cholecystokinin (CCK) stimulates the gall bladder to contract and discharge its bile into the duodenum. Bile contains:

- **bile acids**. These amphiphilic steroids emulsify ingested fat. The hydrophobic portion of the steroid dissolves in the fat while the negatively-charged side chain interacts with water molecules. The mutual repulsion of these negatively-charged droplets

keeps them from coalescing. Thus large globules of fat (liquid at body temperature) are emulsified into tiny droplets (about 1 µm in diameter) that can be more easily digested and absorbed.
- **bile pigments**. These are the products of the breakdown of hemoglobin removed by the liver from old red blood cells. The brownish color of the bile pigments imparts the characteristic brown color of the feces.

The Hepatic Portal System

The capillary beds of most tissues drain into veins that lead directly back to the heart. But blood draining the intestines is an exception. The veins draining the intestine lead to a second set of capillary beds in the liver.

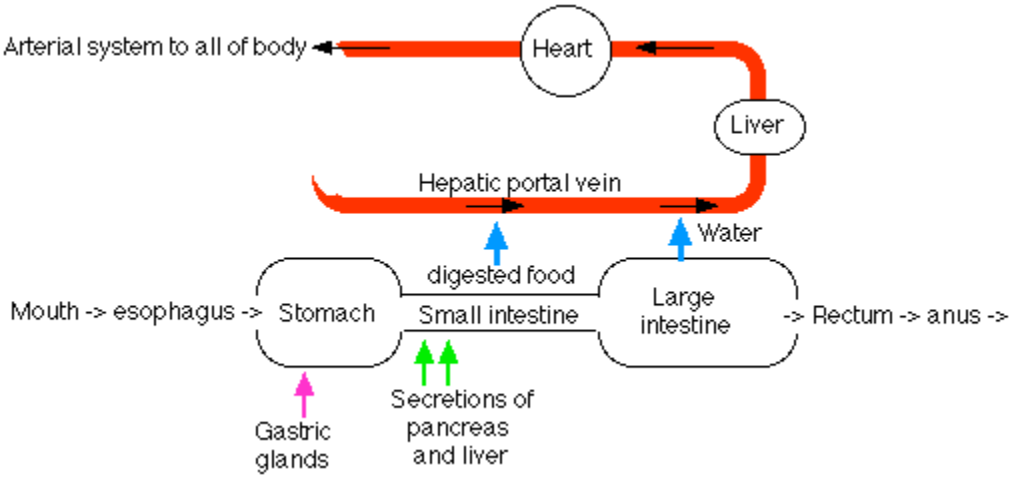


Figure 15.1.1.3 Hepatic portal system

Here the liver removes many of the materials that were absorbed by the intestine:

- Glucose is removed and converted into glycogen.
- Other monosaccharides are removed and converted into glucose.
- Excess amino acids are removed and deaminated.
  - The amino group is converted into **urea**.
  - The residue can then enter the pathways of cellular respiration and be oxidized for energy.
- Many nonnutritive molecules, such as ingested drugs, are removed by the liver and, often, detoxified.

The liver serves as a gatekeeper between the intestines and the general circulation. It screens blood reaching it in the hepatic portal system so that its composition when it leaves will be close to normal for the body. Furthermore, this homeostatic mechanism works both ways. When, for example, the concentration of glucose in the blood drops between meals, the liver releases more to the blood by converting its glycogen stores to glucose (glycogenolysis) and converting certain amino acids into glucose (gluconeogenesis)

The Pancreas

The pancreas consists of clusters of endocrine cells (the islets of Langerhans) and exocrine cells whose secretions drain into the duodenum. Pancreatic fluid contains:

- **sodium bicarbonate** ($NaHCO_3$). This neutralizes the acidity of the fluid arriving from the stomach raising its pH to about 8.
- pancreatic **amylase**. This enzyme hydrolyzes starch into a mixture of maltose and glucose.
- pancreatic **lipase**. The enzyme hydrolyzes ingested fats into a mixture of fatty acids and monoglycerides. Its action is enhanced by the detergent effect of bile.
- 4 **"zymogens"** — proteins that are precursors to active proteases. These are immediately converted into the active proteolytic enzymes:
  - **trypsin.** Trypsin cleaves peptide bonds on the C-terminal side of arginines and lysines.
  - **chymotrypsin.** Chymotrypsin cuts on the C-terminal side of tyrosine, phenylalanine, and tryptophan residues (the same bonds as pepsin, whose action ceases when the $NaHCO_3$ raises the pH of the intestinal contents).

    - **elastase.** Elastase cuts peptide bonds next to small, uncharged side chains such as those of alanine and serine.
    - **carboxypeptidase.** This enzyme removes, one by one, the amino acids at the C-terminal of peptides.
- **nucleases**. These hydrolyze ingested nucleic acids (RNA and DNA) into their component nucleotides.

The secretion of pancreatic fluid is controlled by two hormones: **secretin**, which mainly affects the release of sodium bicarbonate and **cholecystokinin** (**CCK**), which stimulates the release of the digestive enzymes

The Small Intestine

Digestion within the small intestine produces a mixture of disaccharides, peptides, fatty acids, and monoglycerides. The final digestion and absorption of these substances occurs in the villi, which line the inner surface of the small intestine. This scanning electron micrograph shows the villi carpeting the inner surface of the small intestine.

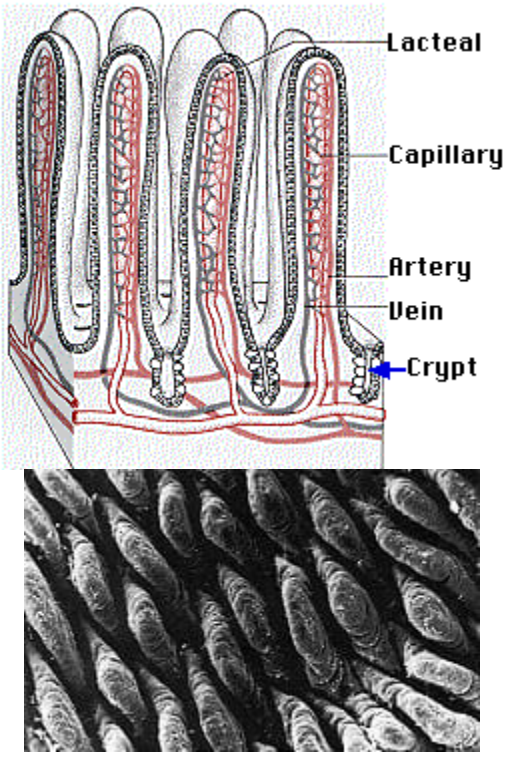


Figure 15.1.1.4 Villi and electron micrograph of villi (courtesy of Keith R. Porter)

The **crypts** at the base of the villi contain **stem cells** that continuously divide by mitosis producing

- More stem cells
- **Paneth cells**, which secrete antimicrobial peptides that suppress the concentration of bacteria in the small intestine
- Cells that migrate up the surface of the villus while differentiating into
    - columnar epithelial cells (the majority),which are responsible for digestion and absorption
    - **goblet cells**, which secrete mucus
    - **endocrine cells**, which secrete a variety of hormones

The continuous production of new epithelial cells replace older cells that after about 5 days die by apoptosis. The villi increase the surface area of the small intestine to many times what it would be if it were simply a tube with smooth walls. In addition, the apical (exposed) surface of the epithelial cells of each villus is covered with **microvilli** (also known as a "brush border"). Thanks largely to these, the total surface area of the intestine is almost 200 square meters, about the size of the singles area of a tennis court and some 100 times the surface area of the exterior of the body.

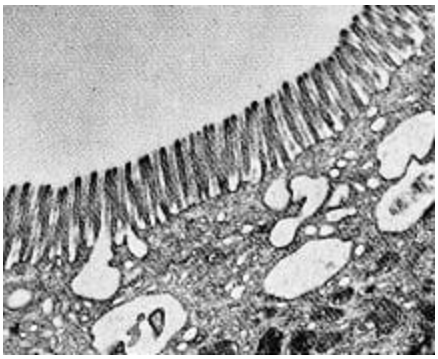


Figure 15.1.1.5: Microvilli

The electron micrograph (courtesy of Dr. Sam L. Clark) shows the microvilli of a mouse intestinal cell. Incorporated in the plasma membrane of the microvilli are a number of enzymes that complete digestion:

- **aminopeptidases** attack the amino terminal (N-terminal) of peptides producing amino acids
- **disaccharidases** convert disaccharides into their monosaccharide subunits
  - **maltase** hydrolyzes maltose into glucose
  - **sucrase** hydrolyzes sucrose (common table sugar) into glucose and fructose
  - **lactase** hydrolyzes lactose (milk sugar) into glucose and galactose

  Fructose simply diffuses into the villi, but both glucose and galactose are absorbed by active transport
- **fatty acids and monoglycerides**. These become resynthesized into fats as they enter the cells of the villus. The resulting small droplets of fat are then discharged by exocytosis into the lymph vessels, called **lacteals**, draining the villi.

Humans with a rare genetic inability to form microvilli die of starvation.

The Large Intestine (colon)

The large intestine receives the liquid residue after digestion and absorption are complete. This residue consists mostly of water as well as any materials that were not digested. The colon contains an enormous ($\sim 4^{13}$) population of microorganisms. Our bodies consist of about the same number ($\sim 3^{13}$) of cells. Most of the species live there perfectly harmlessly; that is, they are commensals. Some are actually beneficial as they synthesize vitamins and digest polysaccharides for which we have no enzymes (providing an estimated 10% of the calories we acquire from our food).

Most of the bacteria belong to the Firmicutes and Bacteroidetes (although used as an indicator of water pollution by feces, E. coli is actually a minor component). In both obese mice (*ob/ob*) and humans, the relative proportion of Bacteroidetes declines and, in mice at least, the efficiency with which residual food is absorbed increases. Putting humans on a diet causes them to regain the normal proportion of Bacteroidetes. Why this relationships exists remains to be discovered. Bacteria flourish to such an extent that as much as 50% of the dry weight of the feces may consist of bacterial cells.

Reabsorption of water is the chief function of the large intestine. The large amounts of water secreted into the stomach and small intestine by the various digestive glands must be reclaimed to avoid dehydration. If the large intestine becomes irritated, it may discharge its contents before water reabsorption is complete causing **diarrhea**. On the other hand, if the colon retains its contents too long, the fecal matter becomes dried out and compressed into hard masses causing **constipation**.

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15.1B: Metabolism

All living things must have an unceasing supply of energy and matter. The transformation of this energy and matter within the body is called **metabolism**. Catabolism is **destructive metabolism**. Typically, in catabolism, larger organic molecules are broken down into smaller constituents. This usually occurs with the release of energy (usually as ATP). Anabolism is **constructive metabolism**. Typically, in anabolism, small precursor molecules are assembled into larger organic molecules. This always requires the input of energy (often as ATP).

Autotrophic and Heterotrophic Nutrition

Green plants, algae, and some bacteria are **autotrophs** ("self-feeders"). Most of them use the energy of sunlight to assemble inorganic precursors, chiefly carbon dioxide and water, into the array of organic macromolecules of which they are made. The process is **photosynthesis**. Photosynthesis makes the ATP needed for the anabolic reactions in the cell.

All other organisms, including ourselves, are **heterotrophs**. We secure all our energy from organic molecules taken in from our surroundings ("food"). Although heterotrophs may feed partially (as most of us do) or exclusively on other heterotrophs, all the food molecules come ultimately from autotrophs. We may eat beef but the steer ate grass. Heterotrophs degrade some of the organic molecules they take in (catabolism) to make the ATP that they need to synthesize the others into the macromolecules of which they are made (anabolism).

How humans (and other animals) do it

Humans are heterotrophs. We are totally dependent on ingested preformed organic molecules to meet all our **energy** needs. We are also dependent on preformed organic molecules as the building blocks to meet our **anabolic** needs.

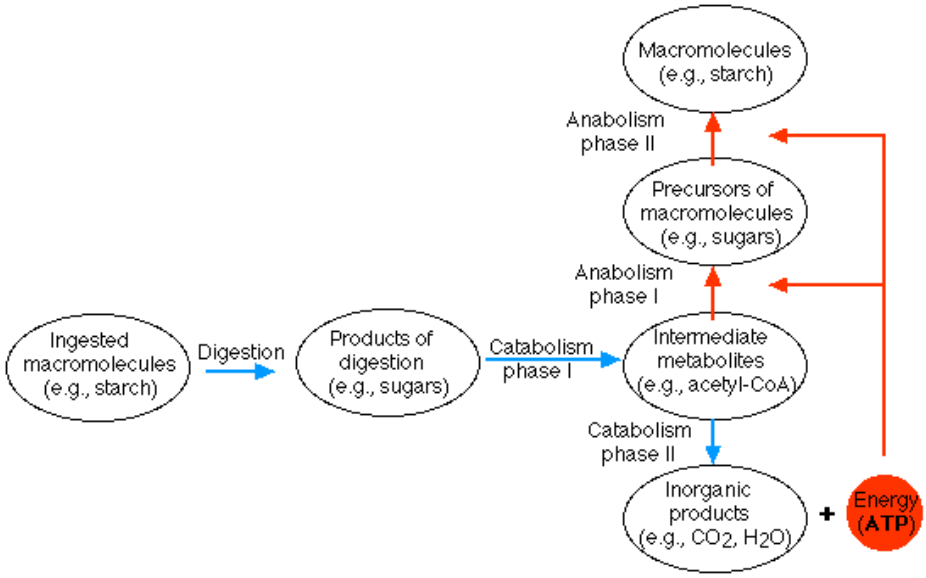


Figure 15.1.2.1 Metabolic pathways

- **Ingestion**: taking food within the body (although as the figure shows, it is still topologically in the external world, not the internal).
- **Digestion**.
  The enzyme-catalyzed hydrolysis of
  - polysaccharides (e.g., starch) to sugars
  - proteins to amino acids
  - fats to fatty acids and glycerol
  - nucleic acids to nucleotides

- **Absorption** into the body and transport to the cells.
- Absorption into cells

Within cells, these molecules are further degraded into still simpler molecules containing two to four carbon atoms. These fragments (**acetyl-CoA** for example) face one of two alternatives:

- They may proceed up various metabolic pathways and serve as the building blocks of, for example, sugars and fatty acids. From these will be assembled the macromolecules of the cell:
  - polysaccharides
  - fats
  - proteins
  - nucleic acids
- Or the molecules in this pool of two- to four-carbon fragments may be still further degraded — ultimately to simple inorganic molecules such as carbon dioxide (CO_2), H_2O, and ammonia (NH_3). This phase of catabolism releases large amounts of energy (in the form of **ATP**). One use to which this energy is put is to run the anabolic activities of the cell.

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15.1C: Nutrition

The human diet must provide the following:

- **calories -** enough to meet our daily energy needs
- **amino acids - t**here are nine, or so, "essential" amino acids that we need for protein synthesis and that we cannot synthesize from other precursors
- **fatty acids** - there are three "essential" fatty acids that we cannot synthesize from other precursors
- **minerals -** inorganic ions generally 18 different ones, calcium in relatively large amounts, zinc in "trace" amounts
- **vitamins** - a dozen or so, small organic molecules that we cannot synthesize from other precursors in our diet

Determining what substances must be incorporated in the human diet and how much of each is incorporated even after years of research still under active study. Why the uncertainty? Inadequate intake of some vitamins produces easily-recognized deficiency diseases like However, it is so difficult to exclude some other possible vitamins from the diet that deficiency diseases are hard to demonstrate.

- **scurvy**: lack of ascorbic acid (vitamin C)
- **beriberi**: lack of thiamine (vitamin B1)
- **pellagra**: lack of niacin

Similarly, some minerals are needed is such vanishingly small amounts that it is practically impossible to prepare a diet that does not include them. However, totally synthetic diets are now available for intravenous feeding of people who cannot eat. This so-called **total parenteral nutrition** has revealed, unexpectedly, some additional trace element needs: chromium and molybdenum.

Despite some uncertainties, the Food and Nutrition Board of the U. S. National Academy of Sciences publishes guidelines. One of the most useful of these is called **recommended daily allowances** or **RDA**s. These provide the basis for the nutrition labels on food.

Carbohydrates

Carbohydrates provide the bulk of the calories (4 kcal/gram) in most diets and starches provide the bulk of that. Age, sex, size, health, and the intensity of physical activity strongly affect the daily need for calories. Moderately active females (19–30 years old) need 1500–2500 kcal/day, while males of the same age need 2500–3300 kcal/day. In some poor countries, too many children do not receive enough calories to grow properly. In order to maintain blood sugar levels, they attack their own protein. This condition of semi-starvation is known as **marasmus**.

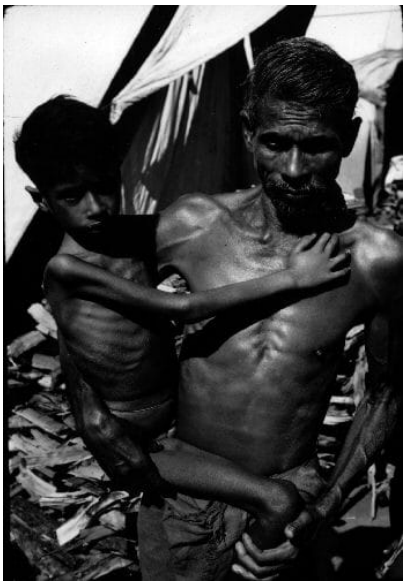


Figure 1: Child suffering with Marasmus in India. (public domain; CDC/ Don Eddins)

Protein

Humans must include adequate amounts of 9 amino acids in their diet. These "essential" amino acids cannot be synthesized from other precursors. However, cysteine can partially meet the need for methionine (they both contain sulfur), and tyrosine can partially substitute for phenylalanine.

The Essential Amino Acids for Humans

Histidine
Isoleucine
Leucine
Lysine
Methionine (and/or cysteine)
Phenylalanine (and/or tyrosine)
Threonine
Tryptophan
Valine

Two of the essential amino acids, **lysine** and **tryptophan**, are poorly represented in most plant proteins. Thus strict vegetarians should take special pains to ensure that their diet contains sufficient amounts of these two amino acids. Birds, mammals, and some other animals are able to discriminate food that contains a nutrient, e.g., an essential amino acid, that they need from food that doesn't. If offered a food lacking that nutrient, they quickly stop eating it. How is this done? In rats, at least, it turns out that certain neurons in the brain detect the lack of an essential amino acid and signal the appetite centers of the brain to stop feeding on deficient food. The neurons detect the lack by the failure of their transfer RNAs (tRNAs) for that amino acid to acquire it. Rats whose tRNAs for threonine have been blocked from loading threonine cease feeding even if their food contains adequate concentrations of it.

Fats

Ingested fats provide the precursors from which we synthesize our own fat as well as cholesterol and various phospholipids. Fat provides our most concentrated form of energy. Its energy content (9 kcal/gram) is over twice as great as carbohydrates and proteins (4 kcal/gram). Humans can synthesize fat from carbohydrates (as most of us know all too well). However, three essential

fatty acids cannot be synthesized this way and must be incorporated in the diet. These are

- linoleic acid
- linolenic acid
- arachidonic acid

All are unsaturated; that is, have double bonds.

Types of fats

- **Saturated**. No double bonds between the carbon atoms in the fatty acid chains. Most animal fats (e.g., butter) are highly saturated.
- **Monounsaturated**. Have a single double bond in the fatty acid chains. Examples are olive, peanut, and rapeseed (canola) oil.
- **Polyunsaturated**. Have two or more double bonds in their fatty acid chains. Examples: corn, soy bean, cottonseed, sunflower, and safflower oils.
- **Trans Fats**. Have been partially hydrogenated producing fewer double bonds and of those that remain, converting them from a cis to a trans configuration.
- **Omega-3 fats**. Have at least one double bond three carbon atoms in from the end of the fatty acid molecule. Linolenic acid is an example. Fish oils are a rich source of omega-3 fatty acids.

Many studies have examined the relationship between fat in the diet and cardiovascular disease. There is still no consensus, but the evidence seems to indicate that Mono and polyunsaturated fats are less harmful than saturated ones, **except that trans unsaturated fats may be worse than saturated fats.** Ingestion of omega-3 unsaturated fats may be protective. For this reason, 1.1 grams/day for women (1.6 for men) is recommended.

Read the label



Figure 15.1.3.1 Nutrition label

At present, food labels in the U.S. list the total amount of fat in a serving of the product (5 g in the example shown here) with a breakdown of the amounts of saturated (1 g), polyunsaturated (0.5 g), and monounsaturated fat (1.5 g).

What about trans fats? There is a proposal to have them included, but at present they are not. However, if you add the amounts of saturated, polyunsaturated, and monounsaturated fat, and the total does not equal "Total Fat" , the discrepancy (2 g in this example) represents the amount of trans fat. Baked goods (like the one whose label is shown here) tend to have quite a bit of trans fat.

Minerals

Calcium

Calcium is essential to almost every function in the body. Blood clotting, intracellular signaling and muscle contraction need only trace amounts. However, large amounts of calcium are needed to make bone (which is 18% calcium), So substantial amounts are needed in the diet, especially during infancy, childhood, and pregnancy. Three hormones parathyroid hormone (PTH), calcitonin and calciferol (vitamin D) work together to regulate how much calcium

- is absorbed from your food
- is taken from, or added to, bone
- is excreted in the urine.

A temporary deficit in the amount of calcium in the diet can be compensated for by its removal from the huge reserves in bone.

Iron (Fe)

Iron is incorporated in a number of body constituents, notably cytochromes, myoglobin and **hemoglobin**. Not surprisingly, an iron deficiency shows up first as **anemia**.

In developed countries like the U.S., iron deficiency is the most common mineral deficiency. It is particularly common among women because of the loss of blood during menstruation and the need for extra iron during pregnancy and breast feeding.

Marginal iron intake is so widespread that some nutritionists want to have iron added to common foods like bread and cereals, just as some vitamins now are. However, excess iron in the body also leads to problems, and this has made the proposal controversial. Even iron supplement tablets pose risks: thousands of children in the U.S. are accidentally poisoned each year by swallowing too many iron tablets. In fact, iron is the most frequent cause of poisoning deaths among children in the U.S.

Iodine

- Incorporated in the hormones thyroxine (T4) and triiodothyronine (T3).
- In regions with iodine-deficient soils, food may not contain enough iodine to meet body needs. The result is **goiter**: a swelling of the thyroid gland.
- The use of iodized salt (table salt to which a small amount of sodium iodide, KI, is added) has reduced the incidence of goiter in most developed countries.

Because iodine deficiency during pregnancy can lead to mental retardation of the infant, it is recommended that pregnant women receive 150–250 µg of iodine daily during both pregnancy and lactation. Hundreds of supplements — both prescription and nonprescription — are sold for this purpose. However, a study of 60 of them reported in the 2/26/09 issue of The New England Journal of Medicine found that only 9 of the 60 contained an amount of iodine within 5% of the amount claimed on the label. Others ranged from only 11% of the amount claimed to almost 3 times as much. Examples: one (prescription) preparation claiming a daily dose of 150 µg actually provided only 26 µg while another (nonprescription) preparation claiming 226 µg of iodine actually contained 610 µg!

Fluoride

The value of fluoride (in ionized form, F^-) was first recognized as a preventive for dental caries (cavities). This makes sense because fluoride ions are incorporated along with calcium and phosphate ions in the crystalline structure of which both bones and teeth are constructed. But it may have other functions.

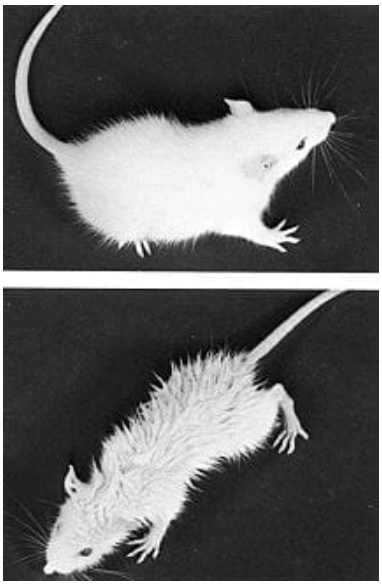


Figure 15.1.3.2 Effect of fluoride on rats

In order to grow properly, a rat must consume 0.5 parts per million (ppm) of fluoride ions in its diet. The rat in the bottom photo received the same diet as that in the top except that tin, vanadium, and fluorides were carefully excluded for 20 days. When tin and vanadium were then given to the deprived rat, it still did not grow normally. But adding 0.5 ppm of potassium fluoride (KF) to its diet restored normal growth and health. (Photos courtesy of Klaus Schwarz, VA Hospital, Long Beach, CA.)

Humans get most of their fluoride in drinking water. In regions where the natural amount is less than 1 ppm, many communities add enough fluoride to bring the concentration up to 1 ppm. Perhaps because the range between optimum and excess is more narrow for fluoride than for most minerals in the diet, water fluoridation has been controversial. Leaving aside the philosophical and political questions raised by proponents and opponents of fluoridation, the safety and efficacy of this public health measure has been thoroughly established.

Zinc

Zinc is incorporated in many enzymes and transcription factors. Zinc supplements are popular for their supposed antioxidant properties and to hasten the recovery from colds. Excessive intake of zinc causes a brief illness. Its most frequent cause is from ingested acidic food or drink that has been stored in galvanized (zinc-coated) containers.

Vitamins

Vitamin A (Retinol)

- **Functions:** Multiple, including serving as the precursor to retinal, the prosthetic group of all four of the light-absorbing pigments in the eye and regulating gene expression essential for the health of epithelia.
- **Sources:** cream, butter, fish liver oils, eggs. Carrots and some other vegetables provide **beta-carotene**, which the liver can convert into vitamin A.
- **Deficiency:** night-blindness.
- **Excess:** stored in the liver, but can be toxic in large doses, especially in children. Even in adults the range between too little and too much is narrow: ingesting vitamin A in amounts not much greater than the recommended dietary allowance (RDA) leads to an increase in bone fractures later in life. High doses taken early in pregnancy have been linked to a greater risk of birth defects. (Its chemical relative isotretinoin — the acne treatment Accutane® — is such a notorious teratogen that it should not be used when there is any chance of a pregnancy occurring).

Thiamine (Vitamin B_1)

- **Function:** coenzyme in cellular respiration.

- **Sources:** meat, yeast, unpolished cereal grains, enriched bread and breakfast cereals.
- **Deficiency:** beriberi. Rarely found in developed countries except among alcoholics.
- **Excess:** water soluble and any excess easily excreted.

Riboflavin (Vitamin B_2)

- **Function:** prosthetic group of flavoprotein enzymes, e.g., flavin adenine dinucleotide (FAD) used in cellular respiration.
- **Sources:** liver, eggs, cheese, milk, enriched bread and breakfast cereals.
- **Deficiency:** damage to eyes, mouth, and genitals.
- **Excess:** water soluble and any excess easily excreted.

Niacin (Nicotinic acid or Vitamin B_3)

- **Function:** this member of the B vitamins is a precursor of NAD and NADP.
- **Sources:** meat, yeast, milk, enriched bread and breakfast cereals.
- **Deficiency:** pellagra (producing skin lesions); a risk where corn (maize) is the staple carbohydrate.
- **Excess:** accidental ingestion of very high doses produces a brief illness, but niacin is water-soluble and any excess is quickly excreted.

Biotin (Vitamin B_7)

- **Function:** this member of the B vitamins is a cofactor in many essential metabolic enzymes.
- **Sources:** liver, egg yolks, corn (maize), intestinal bacteria.
- **Deficiency:** rare except perhaps during pregnancy.
- **Excess:** none identified.

Vitamin B_{12}

- **Function:** needed for DNA synthesis.
- **Sources:** liver, eggs, milk; needs intrinsic factor to be absorbed.
- **Deficiency:** pernicious anemia; caused by lack of intrinsic factor or a vegan diet.
- **Excess:** none identified.

Folic acid (Folacin)

- **Function:** synthesis of purines and pyrimidines.
- **Sources:** green leafy vegetables, but destroyed by cooking.
- **Deficiency:** anemia, birth defects. Women who expect to become pregnant should be extra careful that they receive adequate amounts (400 µg/day). Starting 1 January 1998, any bread or breakfast cereal described as "enriched" must have enough folic acid added to it so that a single serving will provide 10% of this requirement.
- **Excess:** water soluble and any excess easily excreted.

Vitamin C (Ascorbic acid)

- **Functions:** coenzyme in the synthesis of collagen.
- **Sources:** citrus fruits, green peppers, tomatoes; destroyed by cooking.
- **Deficiency:** scurvy.
- **Excess:** Many people take huge amounts of vitamin C, hoping to ward off colds, cancer, etc. They seem to suffer no harm except, perhaps, to their wallets.

Vitamin D

- **Functions:** absorption of calcium from the intestine and bone formation.
- **Sources:**

    - synthesized when ultraviolet light (mostly UV-B) strikes the skin (forms vitamin D3).
    - present in some fish (e.g., salmon), cod liver oil, eggs, and steroid-containing foods irradiated with ultraviolet light.
- **Deficiency:**
    - **rickets** — inadequate conversion of cartilage to bone — in children;
    - osteomalacia — softening of the bones — in adults.

  Until recently, rickets has been very rare in North America. But the combination of two growing trends
    - breast feeding and
    - protecting children from exposure to the sun

  has caused cases to reappear especially in northern latitudes with their short winter days.

  Breast milk provides less than 20% of the recommended daily dose for infants. Until the infant is old enough to eat foods fortified with vitamin D, many pediatricians recommend vitamin supplements for breast-fed babies.

- **Excess:** However, this fat-soluble vitamin is dangerous in very high doses, especially in infants, causing excessive calcium deposits and mental retardation. So some pediatricians view the use of vitamin D supplements for infants with caution (especially since certain preparations have been found to contain amounts far higher than that listed on the label).

Vitamin E (Tocopherol)

- **Function:** acts as an antioxidant agent in cells.
- **Sources:** vegetable oils, nuts, spinach.
- **Deficiency:** anemia, damage to the retinas.
- **Excess:** high doses may be toxic.

Vitamin K

- **Function:** needed for the synthesis of blood clotting factors.
- **Sources:** spinach and other green leafy vegetables; synthesized by intestinal bacteria.
- **Deficiency:** slow clotting of blood. Because
    - little or no vitamin K crosses the placenta,
    - the colon of newborn babies has not yet been colonized by vitamin K-synthesizing bacteria,
    - breast milk is a poor source of the vitamin,

  babies are routinely given vitamin K at birth to eliminate the risk of uncontrolled bleeding.
- **Excess:** No risk from natural forms of the vitamin (K1 and K2).

"Natural" versus "Synthetic" Vitamins

There is no scientific distinction between them. The thiamine molecule (or any other molecule) is the same entity whether synthesized by a plant or by an organic chemist or whether it is still in plant or animal material or has been extracted and incorporated in a pill.

Control of Food Intake

A complex web of signals controls appetite and the intake of food. These include both nerve signals and hormones - both of which signal centers in the brain - chiefly in the hypothalamus. This table lists some of the hormonal signals that have been identified, their effect on appetite and weight gain. Such complexity probably reflects the need for redundant circuits in such a vital activity as acquiring food. But, it also frustrates the search for treatments to attack the increasing incidence of obesity.

Appetite Stimulants	Appetite Suppressants
Ghrelin	Leptin
Agouti-related protein (AgRP)	α-MSH and β-MSH
Neuropeptide Y (NPY)	β-endorphin

Appetite Stimulants	Appetite Suppressants
Melanin-concentrating hormone (MCH)	Cholecystokinin (CCK)
Anandamide	Incretins
Orexins (also called hypocretins)	Insulin
	Amylin
	Pancreatic polypeptide
	PYY_{3-36}
	Brain-derived neurotrophic factor (BDNF)

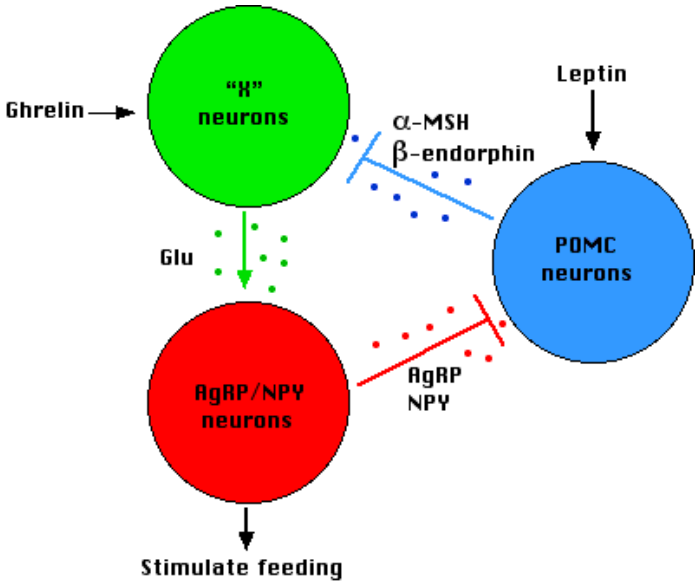


Figure 15.1.3.3 Interaction between hormones

This diagram presents a model of how some of the chief players interact.

- After a period of fasting, secretion of ghrelin activates neurons ("X") in the hypothalamus. They release the excitatory neurotransmitter glutamate where they synapse with AgRP/NPY-releasing neurons. These set in motion the signals that induce feeding.
- A positive feedback loop strengthens the response: AgRP and NPY inhibit the activity of proopiomelanocortin (POMC) neurons whose function is to inhibit "X" neurons (a double-negative is a positive).
- When satiety is finally reached, leptin activates the POMC neurons which release α-MSH and β-endorphin where they synapse with the "X" neurons and the stimulus to continue feeding is stopped. (The precise identity of the "X" neurons remains to be determined.)

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15.1D: Recommended Dietary Allowances

For many years the Food and Nutrition Board of the United States National Academy of Sciences has taken responsibility for establishing guidelines on what quantities of the various nutrients should be eaten by human males and females at various ages. These were called RDAs (for Recommended Dietary Allowances, and often referred to as Recommended Daily Allowances). They provide the data on which food labels are based. In 1997, the Institute of Medicine of the National Academy published a report that added three new categories, including:

- adequate intake ("**AI**"), where no RDA has been established
- tolerable upper intake levels ("**UL**"), to caution against excess intake of nutrients — like vitamin D — that can be harmful in large amounts.

As their findings trickle in, here is a table of RDAs (or AIs) for young adult women and men.

	Females	Males		Females	Males
Protein	46 g	56 g	Folacin	400 µg	same
Vitamin A (retinol)	700 µg*	900 µg*	Biotin	30 µg (AI)	same
Thiamine (Vitamin B_1)	1.1 mg	1.2 mg	Calcium	1000 mg (AI)	same
Riboflavin (Vitamin B_2)	1.1 mg	1.3 mg	Phosphorus	700 mg	same
Niacin (Vitamin B_3)	14 mg	16 mg	Selenium	55 µg	same
Pantothenic acid (Vitamin B_5)	5 mg (AI)	same	Iron	18 mg	8 mg
Vitamin B_6	1.3 mg	same	Zinc	8 mg	11 mg
Vitamin B_{12}	2.4 µg	same	Magnesium	310 mg	400 mg
Vitamin C	75 mg*	90 mg*	Iodine	150 µg	same
Vitamin D	15 µg **	same	Fluoride	3 mg (AI)	4 mg (AI)
Vitamin E	15 mg**	same	Linoleic acid	12 g (AI)	17 g (AI)
Vitamin K	90 µg (AI)	120 µg	α-Linolenic acid	1.1 g (AI)	1.6 g (AI)

*This value has been questioned following the publication of data indicating that a high intake of vitamin A in older people leads to an increased risk of hip fractures. Vitamin A stimulates osteoclasts, the cells that degrade bone, and inhibits osteoblasts, the cells that build bone. To the extent that the vitamin A requirement is met by ingested beta-carotene, these amounts should be multiplied by 12. And that is probably the best way to get your vitamin A as the body only converts enough beta-carotene into vitamin A to meet its needs. There is also evidence that beta-carotene has important functions besides being the precursor of vitamin A and therefore should be ingested in amounts even greater than needed to meet the vitamin A requirement. In short, one should consume vitamin tablets containing beta-carotene and not vitamin A.
*Smokers should add 35 mg to these values, and some nutritionists believe that 200 mg of vitamin C per day is probably optimal for everyone. This is more than twice the current RDA, but far lower than the 2,000 mg/day that is the upper limit (**UL**) and that some people exceed in the hope of warding off colds, cancer, etc.
**15 µg = 600 IU ("International Units").
**The upper limit (UL) is 1,000 mg/day.

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15.2: GAS EXCHANGE



- Topic hierarchy

15.2A: Human Respiratory System

15.2B: Control of Breathing

15.2C: Vertebrate Lungs

15.2D: Tracheal Breathing

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15.2A: Human Respiratory System

The Pathway

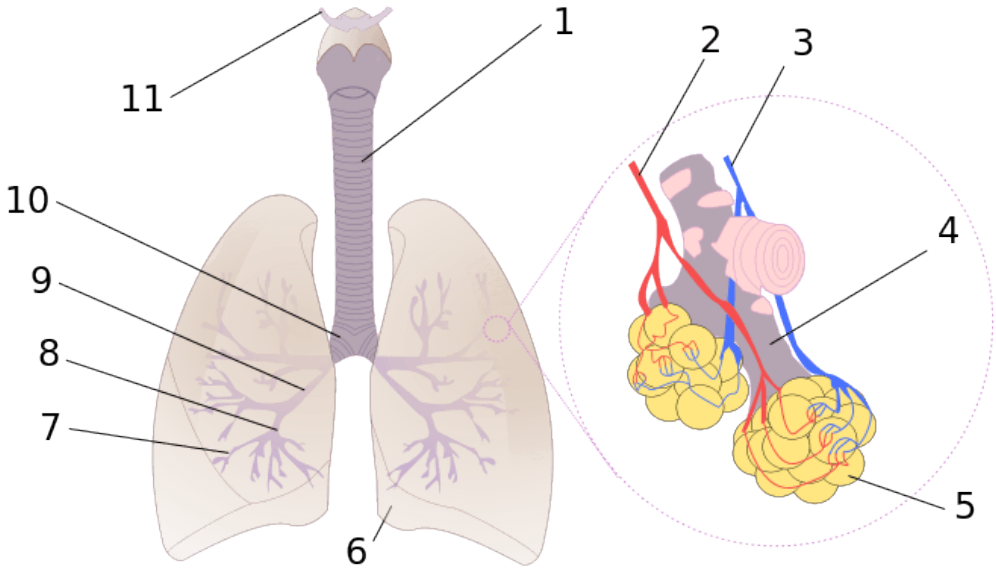


Figure 15.2.1.1: Human lungs. 1:Trachea 2:Pulmonary vein 3:Pulmonary artery 4:Alveolar duct 5:Alveoli 6:Cardiac notch 7:Bronchioles 8:Tertiary bronchi 9:Secondary bronchi 10:Primary bronchi 11:Hyoid bone. (CC-BY-SA-4.0; Credit: **Rastrojo**).

Breathing

In mammals, the diaphragm divides the body cavity into the **abdominal cavity**, which contains the viscera (e.g., stomach and intestines) and the **thoracic cavity**, which contains the heart and lungs. The inner surface of the thoracic cavity and the outer surface of the lungs are lined with **pleural membranes** which adhere to each other. If air is introduced between them, the adhesion is broken and the natural elasticity of the lung causes it to collapse. This can occur from trauma. And it is sometimes induced deliberately to allow the lung to rest. In either case, reinflation occurs as the air is gradually absorbed by the tissues. Because of this adhesion, any action that increases the volume of the thoracic cavity causes the lungs to expand, drawing air into them.

- During inspiration (inhaling), the external intercostal muscles contract, lifting the ribs up and out. This is accomplished by the contraction of the diaphragm muscle, which draws it down. During expiration (exhaling), these processes are reversed and the natural elasticity of the lungs returns them to their normal volume. At rest, we breath 15–18 times a minute exchanging about 500 ml of air.
- In more vigorous expiration, the internal intercostal muscles draw the ribs down and inward and the the wall of the abdomen contracts pushing the stomach and liver upward. Under these conditions, an average adult male can flush his lungs with about 4 liters of air at each breath. This is called the **vital capacity**. Even with maximum expiration, about 1200 ml of **residual air** remain.

The table shows what happens to the composition of air when it reaches the alveoli. Some of the oxygen dissolves in the film of moisture covering the epithelium of the alveoli. From here it diffuses into the blood in a nearby capillary. It enters a red blood cell and combines with the hemoglobin therein. At the same time, some of the carbon dioxide in the blood diffuses into the alveoli from which it can be exhaled.

Table 15.2.1.1: Composition of atmospheric air and expired air in a typical subject. Note that only a fraction of the oxygen inhaled is taken up by the lungs.

Component	Atmospheric Air (%)	Expired Air (%)
N_2 (plus inert gases)	78.62	74.9
O_2	20.85	15.3
CO_2	0.03	3.6
H_2O	0.5	6.2
	100.0%	100.0%

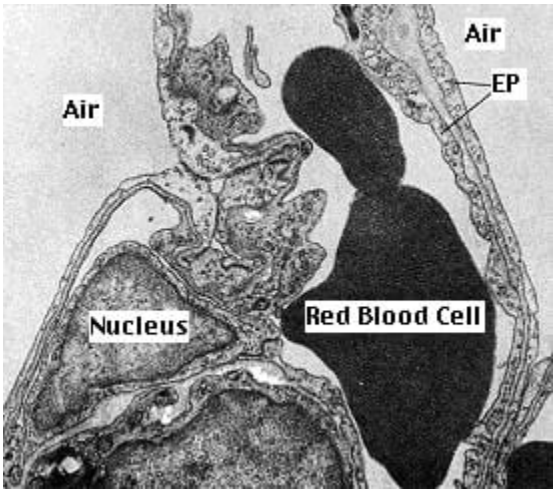


Figure 15.2.1.2: Alveoli(Reproduced with permission from Keith R. Porter and Mary A. Bonneville, **An Introduction to the Fine Structure of Cells and Tissues**, 4th. ed., Lea & Febiger, 1973.)

Central Control of Breathing

The rate of cellular respiration (and hence oxygen consumption and carbon dioxide production) varies with level of activity. Vigorous exercise can increase by 20–25 times the demand of the tissues for oxygen. This is met by increasing the rate and depth of breathing. It is a rising concentration of **carbon dioxide** — not a declining concentration of oxygen — that plays the major role in regulating the ventilation of the lungs. Certain cells in the medulla oblongata are very sensitive to a drop in pH. As the CO_2 content of the blood rises above normal levels, the pH drops

. Those rare people who inherit two defective genes for alpha-1 antitrypsin are particularly susceptible to developing emphysema.

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15.2B: Control of Breathing

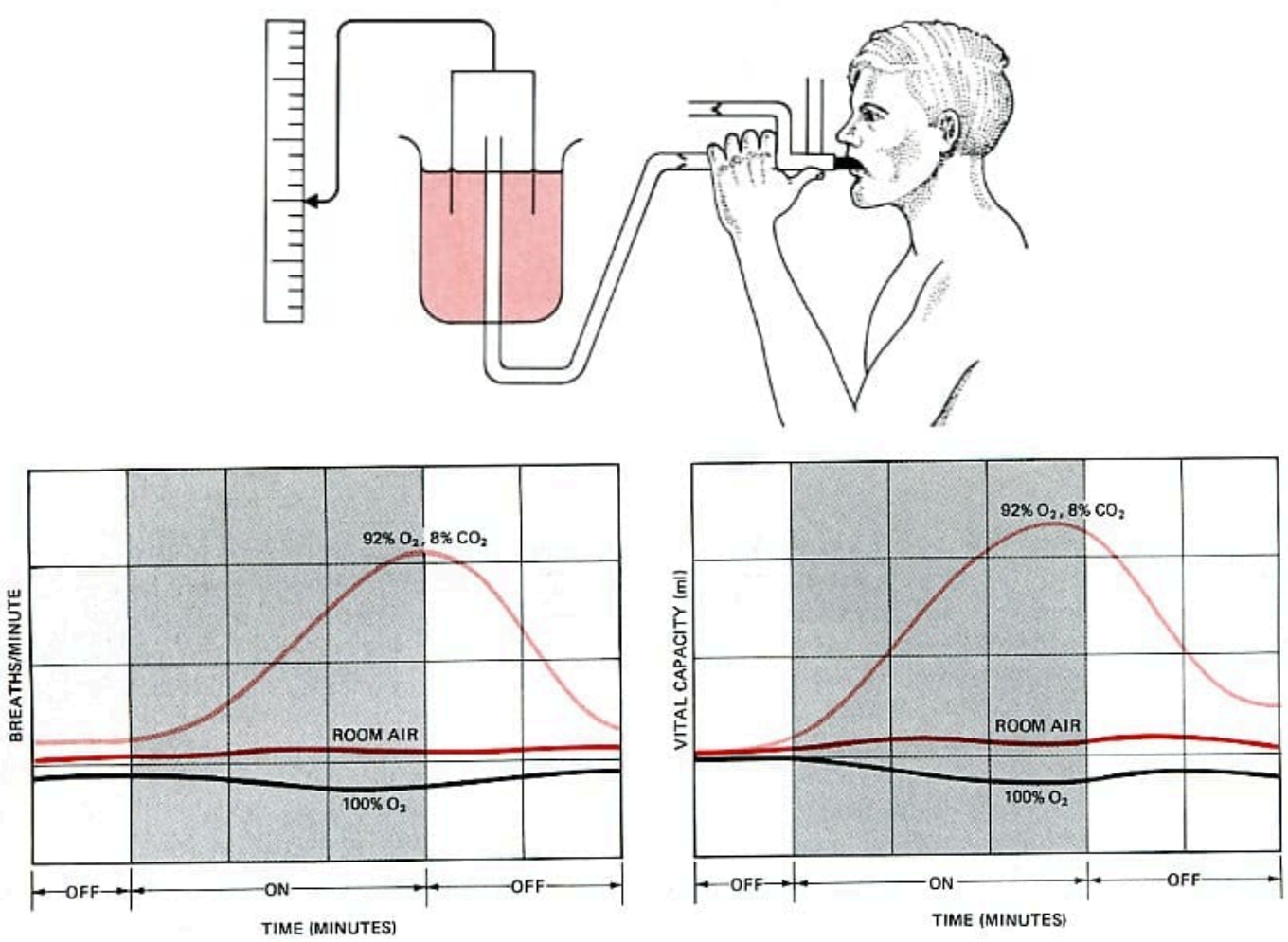


Figure 15.2.2.1 shows an experimental setup (a modification of a device invented by my grandfather, Francis Gano Benedict) with which a human subject can inspire various precise gas mixtures. While the subject is inhaling the gas mixture, the rate and depth of breathing can be recorded.

- The subject begins by breathing pure air (21% oxygen, 0.03% carbon dioxide, and about 79% inert gases by volume), first from the room and then from the tank. This control reveals what, if any, changes in response can be expected just by breathing from the tank (because of an unpleasant taste or increased air resistance, for example). The two graphs show that no appreciable change does occur when breathing air.
- When 100% oxygen is used instead, no marked change in rate ("breaths/minute") or depth ("vital capacity") of breathing occurs either, although there is a tendency for depth of breathing to decrease slightly.
- When the subject inhales a gas mixture consisting of 92% oxygen and 8% carbon dioxide, however, a most dramatic increase in the rate and depth of inspiration takes place. Note that there is no question of tissues lacking oxygen. The gas mixture contains four times as much oxygen as air.

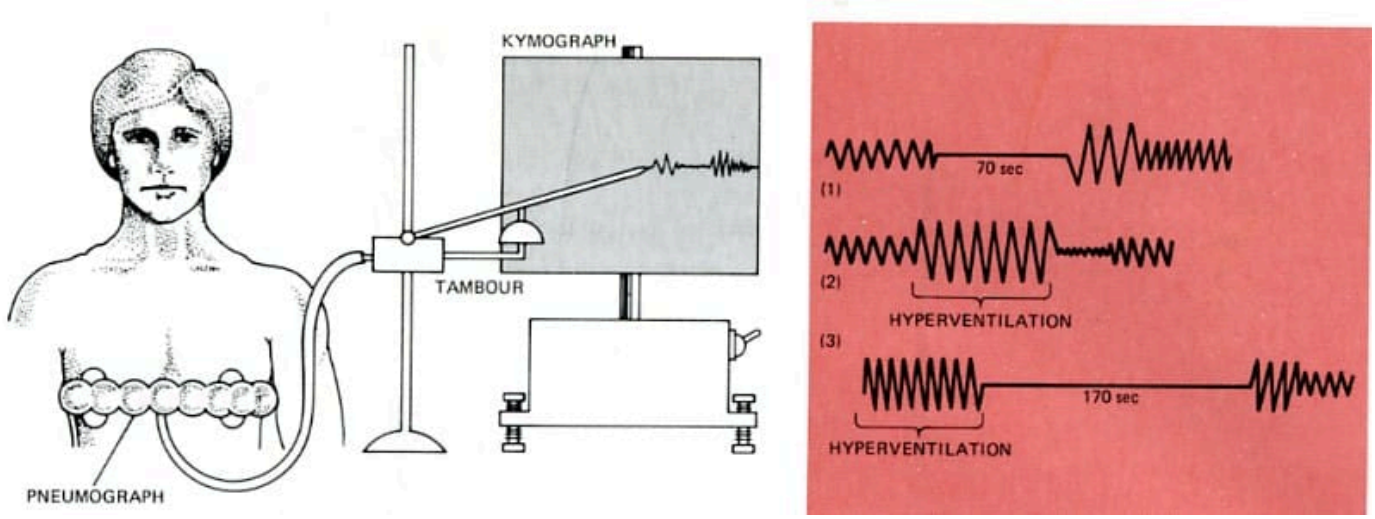


Figure 15.2.2.2 shows a setup with which this type of response can be studied. Movements of the subject's chest are detected by a hollow bellows (the pneumograph) strapped around the chest. Expansion and contraction of the bellows causes decreases and increases in the air within. These pressure changes are transmitted to a recording stylus, which writes on a slowly revolving drum (the kymograph).

- Note that after a period of breath-holding, the rate and depth of inspiration are markedly greater than before the breath-holding began (**1**). This can be explained by the build-up of CO_2 during the breath-holding period.
- Vigorous, forced hyperventilation reduces the CO_2 content of the alveolar air and blood to below its normal value, leading to a period of shallow breathing before its concentration builds back up to normal (**2**).
- The length of time that one can hold his or her breath to the breaking point can be substantially increased by hyperventilating just prior to the period of breath-holding (**3**).

It may seem curious that the rate at which one breathes and thus supplies oxygen to the body is controlled by carbon dioxide rather than oxygen. But cellular respiration produces CO_2 as fast as oxygen is consumed, so the CO_2 given off by active muscles triggers increased ventilation of the lungs and thus automatically supplies additional oxygen. While CO_2 is the major stimulus for controlling breathing, the **carotid body** in the carotid arteries does have receptors that respond to a drop in oxygen. Their activation is important in situations (e.g., at high altitude in the unpressurized cabin of an aircraft) where oxygen supply is inadequate, but there has been no increase in the production of CO_2. People who live at high altitudes, e.g., in the Andes, have enlarged carotid bodies.

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15.2C: Vertebrate Lungs

Terrestrial vertebrates (**amphibians**, **reptiles**, **birds**, and **mammals**) use a pair of lungs to exchange oxygen and carbon dioxide between their tissues and the air.

Frog Lungs

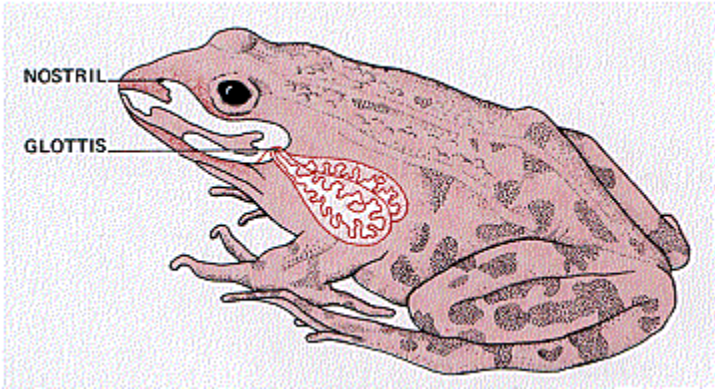


Figure 15.2.3.1 Frog lungs

The frog's lungs are a pair of thin-walled sacs connected to the mouth through an opening, the glottis. The surface area of the lungs is increased by inner partitions which are richly supplied with blood vessels. The frog inflates its lungs by

- filling its mouth with air
- then closing its mouth
- closing the internal openings to its nostrils
- opening its glottis
- raising the floor of its mouth thus forcing air into the lungs.

The frog's skin serves as a supplementary organ of gas exchange. However, it must remain moist to do this, which is one reason that frogs, like other amphibians, live in moist places. The frog's circulatory system, which brings oxygen-depleted blood to its lungs (and skin) and takes oxygen-enriched blood away is described in a separate page.

Reptile Lungs

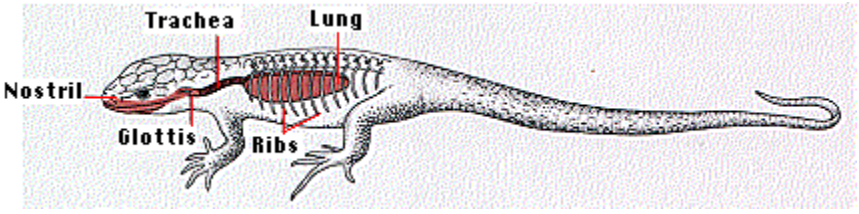


Figure 15.2.3.2 Reptile lungs

The skin of reptiles is dry and scaly, so they can live in arid locations (although many do not). However, they cannot use their skin as an organ of gas exchange. Reptiles depend entirely on their lungs for this. Their lungs are considerably more efficient than those of amphibians.

- They have a much greater surface area for the exchange of gases.
- They are inflated and deflated by the bellowslike expansion and contraction of the rib cage.

While fresh air flows in and stale air out of the lizard's lungs, another reptile, the American alligator, uses a more efficient mechanism similar to that described below in birds.)

The lizard's circulatory system, which brings oxygen-depleted blood to its lungs and takes oxygen-enriched blood away is described in a separate page.

Bird Lungs

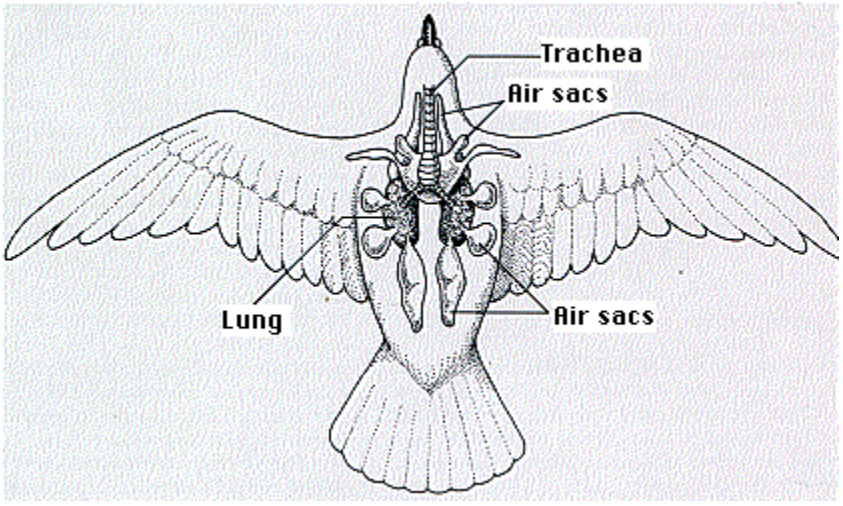


Figure 15.2.3.3 Bird lungs

Unlike reptiles, birds are homeothermic ("warm blooded"), maintaining a constant body temperature (usually around 40°C) despite wide fluctuations in the temperature of their surroundings. They maintain their body temperature with the heat produced by muscular activity. This depends, in turn, on a high rate of cellular respiration. So the demands on the gas-exchange efficiency of the lungs of a small, active bird are great.

Although the ventilation of bird lungs is similar to that of reptiles, their effectiveness is increased by the presence of **air sacs**. Although no gas exchange occurs in the air sacs, their arrangement increases the efficiency of lung ventilation by enabling fresh air to pass in one direction through the lungs during **both** inhalation and exhalation. The air sacs also aid in reducing the density of the body by substituting air for tissue or fluid in many places. Even some of the bird's bones are penetrated by air sacs.

Mammalian Lungs

Ventilation of mammalian lungs is assisted by the **diaphragm** - a muscular partition that divides the thoracic cavity from the abdominal cavity.

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15.2D: Tracheal Breathing

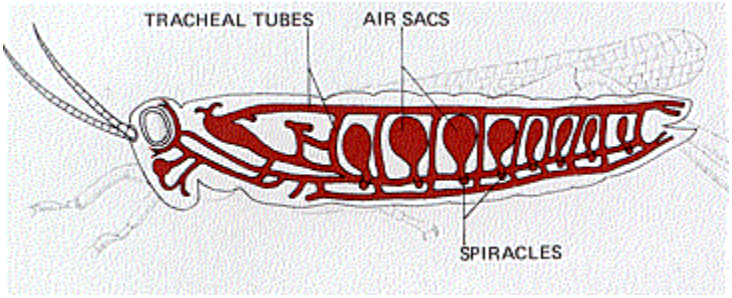


Figure 15.2.4.1 Insect trachea

Tracheae open to the outside through small holes called **spiracles**. In the grasshopper, the first and third segments of the thorax have a spiracle on each side. Another 8 pairs of spiracles are arranged in a line on either side of the abdomen. The spiracles are guarded by

- valves controlled by muscles that enables the grasshopper to open and close them
- hairs that filter out dust as the air enters the spiracles

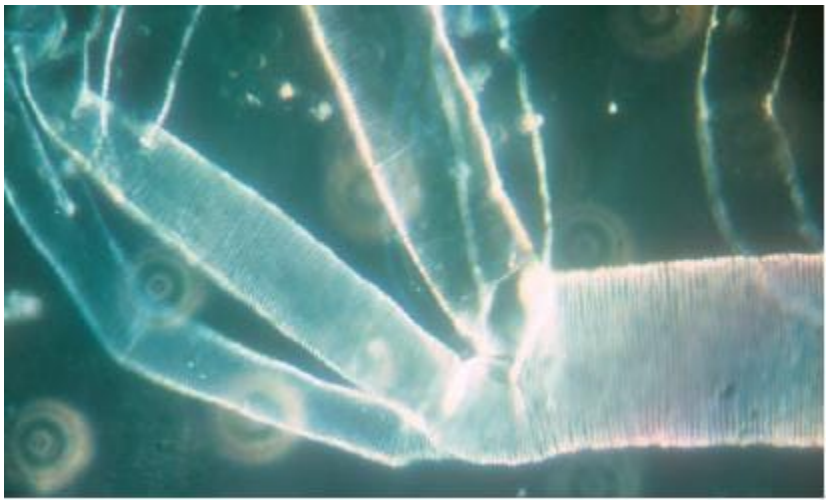


Figure 15.2.4.3 Fraenkel experiment

The experiment illustrated (first performed by the insect physiologist Gottfried Fraenkel) shows that there is a one-way flow of air through the grasshopper. The liquid seals in the tubing move to the right as air enters the spiracles in the thorax and is discharged through the spiracles in the abdomen. The rubber diaphragm seals the thorax from the abdomen. The one-way flow of air increases the efficiency of gas exchange as CO_2-enriched air can be expelled without mingling with the incoming flow of fresh air.

Gas Exchange in Aquatic Insects

Even aquatic insects use a tracheal system for gas exchange.

- Some, like mosquito larvae ("wigglers"), get their air by poking a breathing tube — connected to their tracheal system — through the water surface.
- Some insects that can submerge for long periods carry a bubble of air with them from which they breathe.
- Still others have spiracles mounted on the tips of spines. With these they pierce the leaves of underwater plants and obtain oxygen from the bubbles formed (by photosynthesis) within the leaves.
- Even in aquatic insects that have gills, after oxygen diffuses from the water into the gills, it then diffuses through a gas-filled

tracheal system for transport through the body.

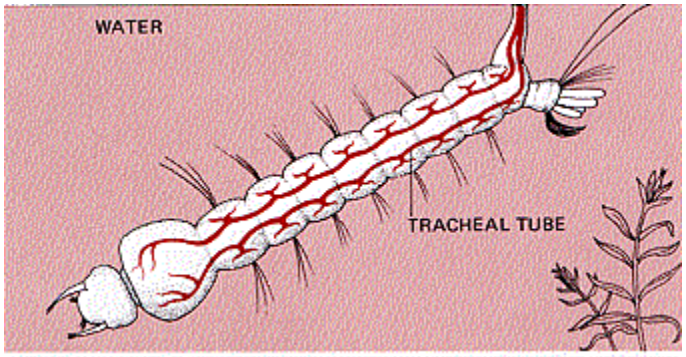


Figure 15.2.4.4 Aquatic animal trachea

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15.3: CIRCULATORY SYSTEMS



- Topic hierarchy

15.3A: Anatomy of Human Circulatory System

15.3B: How the Human Circulatory System Works

15.3C: The Heartbeat

15.3D: The Lymphatic System

15.3E: Blood

15.3F: Blood Groups

15.3G: The Transport of Heat

15.3H: Blood Clotting

15.3I: Sickle-Cell Disease

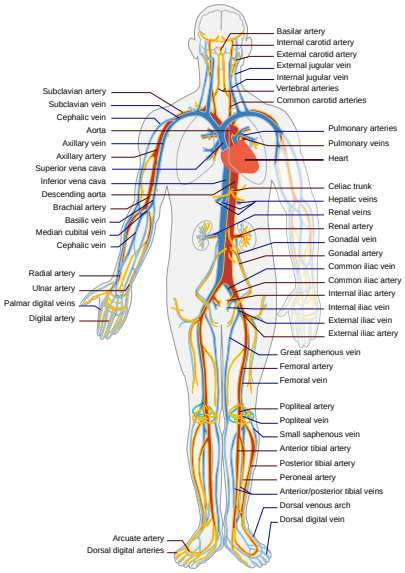
15.3J: Serine Proteases

15.3K: Animal Circulatory Systems

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15.3A: Anatomy of Human Circulatory System

The circulatory system is an organ system that permits blood to circulate and transport nutrients (such as amino acids and electrolytes), oxygen, carbon dioxide, hormones, and blood cells to and from the cells in the body to provide nourishment and help in fighting diseases, stabilize temperature and pH, and maintain homeostasis.



Simplified diagram of the human Circulatory system in anterior view. (Public Domain; LadyofHats)

Main Features of the Human Circulatory System

- A liquid, blood, to transport nutrients, wastes, oxygen and carbon dioxide, and hormones.
- **Two pumps** (in a single **heart**): one to pump **deoxygenated** blood to the lungs and the other to pump **oxygenated** blood to all the other organs and tissues of the body
- A system of **blood vessels** to distribute blood throughout the body
- Specialized **organs for exchange** of materials between the blood and the external environment; for example organs like the lungs and intestine that add materials to the blood and organs like the lungs and kidneys that remove materials from the blood and deposit them back in the external environment

The Heart and Pulmonary System

The heart is located roughly in the center of the chest cavity. It is covered by a protective membrane, the **pericardium**.

- Deoxygenated blood from the body enters the **right atrium**.
- It flows through the **tricuspid valve** into the **right ventricle**. The term tricuspid refers to the three flaps of tissue that make up the valve.
- Contraction of the ventricle then closes the tricuspid valve and forces open the pulmonary valve.
- Blood flows into the **pulmonary artery**.
- This branches immediately, carrying blood to the right and left **lungs**.
- Here the blood gives up carbon dioxide and takes on a fresh supply of oxygen.
- The capillary beds of the lungs are drained by venules that are the tributaries of the **pulmonary veins**.
- Four pulmonary veins, two draining each lung, carry oxygenated blood to the **left atrium** of the heart.

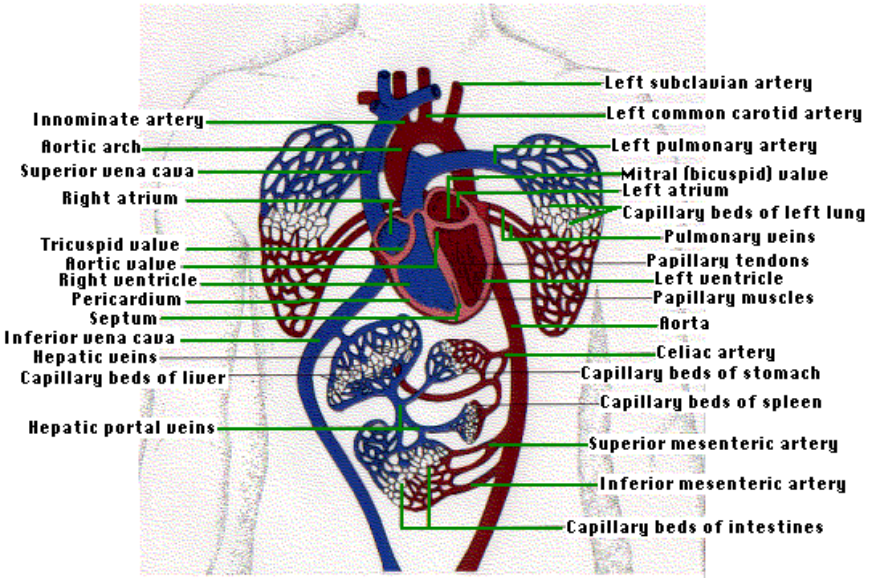


Figure 15.3.1.1 Human heart

The above figure shows the human heart, with a schematic view of the pathway of blood through the lungs and internal organs. Oxygenated blood is shown in red; deoxygenated blood in blue. Note that the blood draining the stomach, spleen, and intestines passes through the liver before it is returned to the heart. Here surplus or harmful materials picked up from those organs can be removed before the blood returns to the general circulation.

The Coronary System

From the **left atrium**,

- Blood flows through the **mitral valve** (also known as the bicuspid valve) into the **left ventricle**.
- Contraction of the ventricle closes the mitral valve and opens the **aortic valve** at the entrance to the **aorta**.
- The first branches from the aorta occur just beyond the aortic valve still within the heart.
- Two openings lead to the right and left **coronary arteries**, which supply blood to the heart itself. Although the coronary arteries arise within the heart, they pass directly out to the surface of the heart and extend down across it. They supply blood to the network of capillaries that penetrate every portion of the heart.
- The capillaries drain into two **coronary veins** that empty into the **right atrium**.

Diseases of the Coronary system: Arteriosclerosis and Atherosclerosis

The coronary arteries arise at the point of maximum blood pressure in the circulatory system. Over the course of time, the arterial walls are apt to lose elasticity, which limits the amount of blood that can surge through them and hence limits the supply of oxygen to the heart. This condition is known as *arteriosclerosis*.

Alternatively, fatty deposits, called plaque, may accumulate on the interior surface of the coronary arteries; this condition is known as *atherosclerosis*. This is particularly common in people who have high levels of cholesterol in their blood. Plaque deposits reduce the bore of the coronary arteries and thus the amount of blood they can carry. Atherosclerosis (usually along with arteriosclerosis) may limit the blood supply to the heart that during times of stress the heart muscle is so deprived of oxygen that the pain of **angina** is created. It triggers the formation of a clot causing a **coronary thrombosis**. This stops the flow of blood through the vessel and the capillary network it supplies causing a **heart attack**. The portion of the heart muscle deprived of oxygen dies quickly of oxygen starvation. If the area is not too large, the undamaged part of the heart can, in time, compensate for the damage.

Coronary bypass surgery uses segments of leg veins to bypass the clogged portions of the coronary arteries.

The Systemic Circulation

The remainder of the system is known as the systemic circulation. The graphic shows the major arteries (in bright red) and veins (dark red) of the system. Blood from the aorta passes into a branching system of arteries that lead to all parts of the body. It then flows into a system of capillaries where its exchange functions take place.

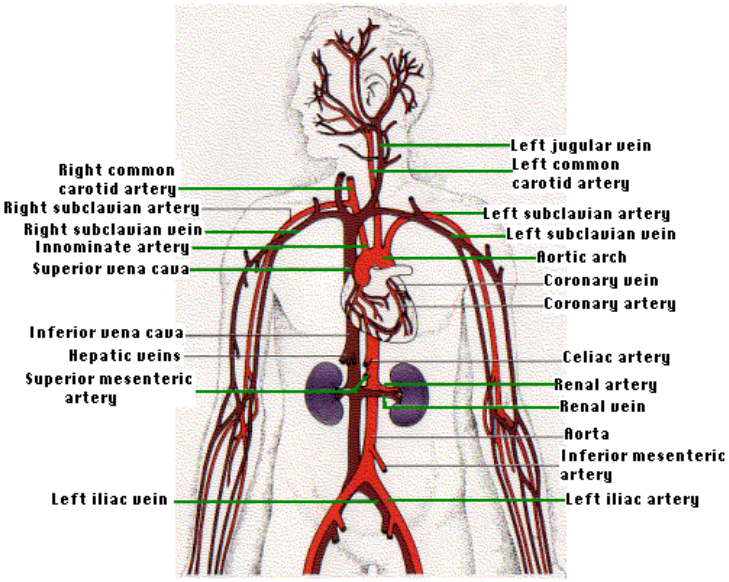


Figure 15.3.1.2 Human circulation system

Blood from the capillaries flows into venules which are drained by veins.

- Veins draining the upper portion of the body lead to the **superior vena cava**.
- Veins draining the lower part of the body lead to the **inferior vena cava**.
- Both empty into the right atrium.

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15.3B: How the Human Circulatory System Works

All the functions of the circulatory system occur in the capillary beds. The rest of the system consists of two pumps (in the heart) and associated plumbing:

- **arteries** and their terminal branches, the **arterioles**
- **veins** and their tributaries, the **venules**.

Blood Pressure

Blood moves through the arteries, arterioles, and capillaries because of the force created by the contraction of the ventricles.

Blood pressure in the arteries.

The surge of blood that occurs at each contraction is transmitted through the elastic walls of the entire arterial system where it can be detected as the **pulse**. Even during the brief interval when the heart is relaxed — called **diastole** — there is still pressure in the arteries. When the heart contracts — called **systole** — the pressure increases.

Blood pressure is expressed as two numbers, e.g., 120/80. The first is the pressure during systole. The unit of measure is the **torr**, in this example, the pressure equivalent to that produced by a column of mercury 120 mm high. The second number is the pressure at diastole.

Although blood pressure can vary greatly in an individual, continual high pressure — especially diastolic pressure — may be the symptom or cause of a variety of ailments. The medical term for high blood pressure is **hypertension**.

Blood pressure in the capillaries

The pressure of arterial blood is largely dissipated when the blood enters the capillaries. Capillaries are tiny vessels with a diameter just about that of a red blood cell (7.5 µm). Although the diameter of a single capillary is quite small, the number of capillaries supplied by a single arteriole is so great that the total cross-sectional area available for the flow of blood is increased. Therefore, the pressure of the blood as it enters the capillaries decreases.

Blood pressure in the veins

When blood leaves the capillaries and enters the venules and veins, little pressure remains to force it along. Blood in the veins below the heart is helped back up to the heart by the **muscle pump**. This is simply the squeezing effect of contracting muscles on the veins running through them. One-way flow to the heart is achieved by valves within the veins.

Exchanges Between Blood and Cells

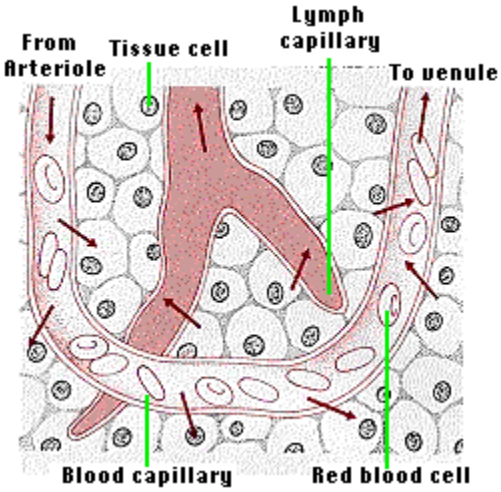


Figure 15.3.2.1 Tissue space

With rare exceptions, our blood does not come into direct contact with the cells it nourishes. As blood enters the capillaries surrounding a **tissue space**, a large fraction of it is filtered into the tissue space. It is this **interstitial** or **extracellular fluid** (**ECF**) that brings to cells all of their requirements and takes away their products. The number and distribution of capillaries is such that probably no cell is ever farther away than 50 µm from a capillary.

When blood enters the arteriole end of a capillary, it is still under pressure (about 35 torr) produced by the contraction of the ventricle. As a result of this pressure, a substantial amount of water and some plasma proteins filter through the walls of the capillaries into the **tissue space**. Thus fluid, called **interstitial fluid**, is simply blood plasma minus most of the proteins. It has the same composition and is formed in the same way as the nephric filtrate in kidneys. Interstitial fluid bathes the cells in the tissue space and substances in it can enter the cells by diffusion or active transport. Substances, like carbon dioxide, can diffuse out of cells and into the interstitial fluid.

Near the venous end of a capillary, the blood pressure is greatly reduced (to about 15 torr). Here another force comes into play. Although the composition of interstitial fluid is similar to that of blood plasma, it contains a smaller concentration of proteins than plasma and thus a somewhat greater concentration of water. This difference sets up an osmotic pressure. Although the osmotic pressure is small (~ 25 torr), it is greater than the blood pressure at the venous end of the capillary. Consequently, the fluid reenters the capillary here.

The first of the four graphs (a) shows this balanced relationship in the capillary bed; the others show what happens when the system is altered.

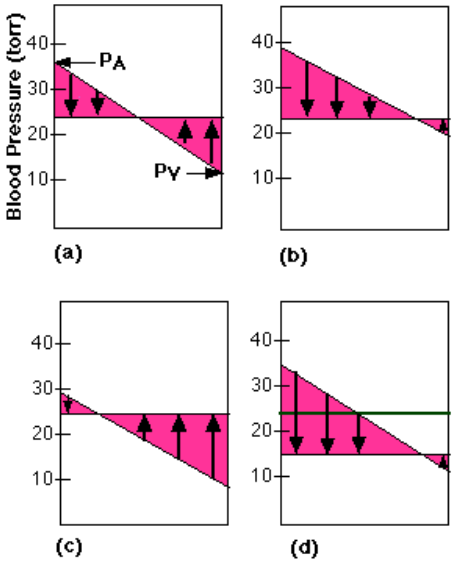


Figure 15.3.2.2 Pressure relations in capillaries

Pressure relations in the capillaries.

P_A = blood pressure at the arteriole end of the capillary.
P_V = blood pressure at the venule end of the capillary.
The horizontal line represents the osmotic pressure of the blood.
When the blood pressure is greater than the osmotic pressure, filtration of interstitial fluid occurs (downward-pointing arrows).
When the blood pressure is less than the osmotic pressure, reabsorption of interstitial fluid occurs (up arrows).

(a) The normal situation. Filtration and absorption are balance.

(b) Result of dilating the arterioles. P_A increases and the tissue space becomes engorged with interstitial fluid.

(c) Result of constricting the arterioles. P_A decreases and interstitial fluid is withdrawn from the tissue space.

(d) Result of a lowered concentration of protein in the blood (such as occurs during prolonged malnutrition). Because of the reduced osmotic pressure (lower horizontal line), fluid accumulates in the tissue spaces resulting in **edema**.

Control of the Capillary Beds

An adult human has been estimated to have some 60,000 miles (96,560 km) of capillaries with a total surface area of some 800–1000 m^2 (an area greater than three tennis courts). The total volume of this system is roughly 5 liters, the same as the total volume of blood. However, if the heart and major vessels are to be kept filled, all the capillaries cannot be filled at once. So a continual redirection of blood from organ to organ takes place in response to the changing needs of the body. During vigorous exercise, for example, capillary beds in the skeletal muscles open at the expense of those in the viscera. The reverse occurs after a heavy meal.

The table shows the distribution of blood in the human body at rest and during vigorous exercise. Note the increase in blood supply to the working organs (skeletal muscles and heart). The increased blood supply to the skin aids in the dissipation of the heat produced by the muscles. Note also that the blood supply to the brain remains constant. The total blood flow during exercise increases because of a more rapid heartbeat and also a greater volume of blood pumped at each beat.

	Blood Flow ml/min	
	At Rest	**During Strenuous Exercise**

Heart	250	750
Kidneys	1,200	600
Skeletal Muscles	1,000	12,5000
Skin	400	1,900
Viscera	1,400	600
Brain	750	750
Other	600	400
Total	5,600	17,500

The walls of arterioles are encased in smooth muscle. Constriction of arterioles decreases blood flow into the capillary beds they supply while dilation has the opposite effect. In time of danger or other stress, for example, the arterioles supplying the skeletal muscles will be dilated while the bore of those supplying the digestive organs will decrease. These actions are carried out by

- the **autonomic nervous system**
- local controls in the capillary beds

Local Control in the Capillary Beds

- **Nitric oxide** (**NO**) is a potent dilator of arteries and arterioles.
  - When the endothelial cells that line these vessels are stimulated, they synthesize nitric oxide. It quickly diffuses into the muscular walls of the vessels causing them to relax.
  - In addition, as the hemoglobin in red blood cells releases its O_2 in actively-respiring tissues, the lowered pH causes it to also release NO which helps dilate the vessels to meet the increased need of the tissue.

  Nitroglycerine, which is often prescribed to reduce the pain of angina, does so by generating nitric oxide, which relaxes the walls of the arteries and arterioles. The prescription drug **sildenafil citrate** ("**Viagra**") does the same for vessels supplying blood to the penis. The effects of these two drugs are additive and using them together could precipitate a dangerous drop in blood pressure.

- Cells where infection or other damage is occurring release substances like **histamine** that dilate the arterioles and thus increase blood flow in the area.
- In most of the body, the flow of blood through a capillary is controlled by the arteriole supplying it. In the brain, however, another mechanism participates. The degree of contraction of **pericytes**, cells that surround the capillary, also adjusts the flow of blood through the capillary. The changes in brain activity seen by such imaging procedures as fMRI and PET scans are probably influenced by pericyte activity.

Shock

Under some circumstances, capillary beds may open without others closing in compensation. Although the volume of blood remains unchanged, blood pressure declines abruptly as blood pools in the capillary beds. If untreated shock is usually fatal.

Shock can also result from severe bleeding. The heart can only pump as much blood as it receives. If insufficient blood gets back to the heart, its output — and hence blood pressure — drops. The tissues fail to receive enough oxygen. This is especially critical for the brain and the heart itself. To cope with the problem, arterioles constrict and shut down the capillary beds — except those in the brain and heart. This reduces the volume of the system and helps maintain normal blood pressure.

Air-breathing vertebrates that spend long periods under water (e.g., seals, penguins, turtles, and alligators) employ a similar mechanism to ensure that the oxygen supply of the heart and brain is not seriously diminished. When the animal dives, the blood supply to the rest of the body is sharply reduced so that what oxygen remains will be available for those organs needing it most: the brain and heart.

Regulation of Blood Pressure by Hormones

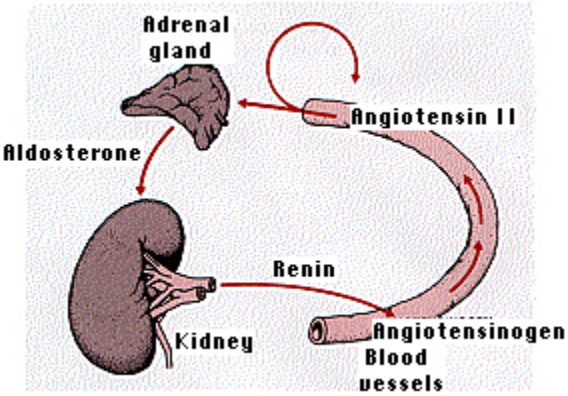


Figure 15.3.2.3 Human kidney

One of the functions of the kidney is to monitor blood pressure and take corrective action if it should drop. The kidney does this by secreting the protease **renin**.

- Renin acts on **angiotensinogen**, a plasma peptide, splitting off a fragment containing 10 amino acids called **angiotensin I**.
- angiotensin I is cleaved by a peptidase secreted by blood vessels called **angiotensin converting enzyme** (**ACE**) producing **angiotensin II**, which contains 8 amino acids.
- angiotensin II
  - constricts the walls of arterioles closing down capillary beds
  - stimulates the proximal tubules in the kidney to reabsorb sodium ions
  - stimulates the adrenal cortex to release **aldosterone**. Aldosterone causes the kidneys to reclaim still more sodium and thus water
  - increases the strength of the heartbeat
  - stimulates the pituitary to release the **vasopressin**

  All of these actions, which are mediated by its binding to G-protein-coupled receptors on the target cells, lead to an **increase in blood pressure**.

The Heart

A rise in blood pressure stretches the atria of the heart. This triggers the release of **atrial natriuretic peptide** (**ANP**). ANP is a peptide of 28 amino acids. ANP lowers blood pressure by:

- relaxing arterioles
- inhibiting the secretion of **renin** and **aldosterone**
- inhibiting the reabsorption of sodium ions in the collecting ducts of the kidneys

The effects on the kidney reduce the reabsorption of water by them thus increasing the flow of urine and the amount of sodium excreted in it (These actions give ANP its name: *natrium* = sodium; *uresis* = urinate). The net effect of these actions is to reduce blood pressure by reducing the volume of blood volume in the system.

Contributors and Attributions

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15.3C: The Heartbeat

During rest, the heart beats about 70 times a minute in the adult male, while pumping about 5 liters of blood.

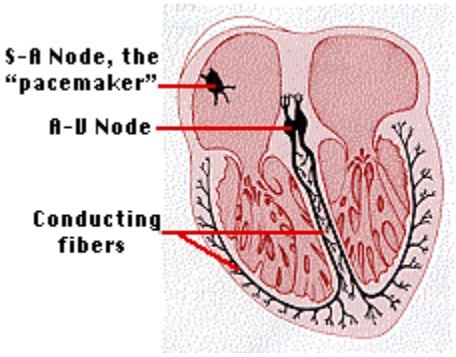


Figure 15.3.3.1 Interior of human heart

The stimulus that maintains this rhythm is self-contained. Embedded in the wall of the **right atrium** is a mass of specialized heart tissue called the sino-atrial (S-A) node. The **S-A node** is also called the **pacemaker** because it establishes the basic frequency at which the heart beats.

The interior of the fibers of heart muscle, like all cells, is negatively charged with respect to the exterior. In the cells of the pacemaker, this charge breaks down spontaneously about 70 times each minute. This, in turn, initiates a similar discharge of the nearby muscle fibers of the atrium. A tiny wave of current sweeps over the atria, causing them to contract.

When this current reaches the region of insulating connective tissue between the atria and the ventricles, it is picked up by the **A-V node** (atrio-ventricular node). This leads to a system of branching fibers that carries the current to all parts of the ventricles. The contraction of the heart in response to this electrical activity creates **systole**. A period of recovery follows called **diastole**.

- The heart muscle and S-A node become recharged.
- The heart muscle relaxes.
- The atria refill.

The Electrocardiogram

The electrical activity of the heart can be detected by electrodes placed at the surface of the body. Analysis of an electrocardiogram (ECG or EKG) aids in determining, for example, the extent of damage following a heart attack. This is because death of a portion of the heart muscle blocks electrical transmission through that area and alters the appearance of the ECG.

Ventricular Fibrillation

The ventricles can maintain a beat even without a functioning A-V node, although the beat is slower. There is, however, a danger that impulses arising in the ventricles may become disorganized and random. If this happens, they begin to twitch spasmodically, a condition called **ventricular fibrillation**. Blood flow ceases and unless the heart rhythm is restarted, death follows swiftly. In fact, ventricular fibrillation is the immediate cause of as much as 25% of all deaths.

Hospital emergency rooms, ambulances, commercial air craft and many other public places are now equipped with **defibrillators** which, by giving the heart a jolt of direct current, may restore its natural rhythm and save the victim's life.

Artificial Pacemakers

These are devices that generate rhythmic impulses that are transmitted to the heart by fine wires. Thanks to miniaturization and long-lived batteries, pacemakers can be implanted just under the skin and reached through a small incision when maintenance is

needed.

Auxiliary Control of the Heart

Although the A-V node sets the basic rhythm of the heart, the **rate** and **strength** of its beating can be modified by two auxiliary control centers located in the medulla oblongata of the brain.

- One sends nerve impulses down **accelerans nerves**.
- The other sends nerve impulses down a pair of **vagus nerves**

The Accelerans Nerve

The accelerans nerve is part of the sympathetic branch of the **autonomic nervous system**, and like all post-ganglionic sympathetic neurons releases **noradrenaline** at its endings on the heart. It increases the rate and strength of the heartbeat and thus increase the flow of blood. Its activation usually arises from some stress such as fear or violent exertion. The heartbeat may increase to 180 beats per minute. The strength of contraction increases as well so the amount of blood pumped may increase to as much as 25–30 liters/minute.

> Note
>
> The 24 Feb 2000 issue of the **New England Journal of Medicine** reports on a family some of whose members have inherited a mutant gene for the transporter that is responsible for reuptake of noradrenaline back into the neuron that released it. Those with the mutation are prone to bouts of rapid heartbeat and fainting when they suddenly stand up.

Vigorous exercise accelerates heartbeat in two ways:

- As cellular respiration increases, so does the carbon dioxide level in the blood. This stimulates receptors in the carotid arteries and aorta, and these transmit impulses to the medulla for relay by the accelerans nerve to the heart.
- As muscular activity increases, the muscle pump drives more blood back to the right atrium. The atrium becomes distended with blood, thus stimulating stretch receptors in its wall. These, too, send impulses to the medulla for relay to the heart.

Distention of the wall of the right atrium also triggers the release of **atrial natriuretic peptide** (**ANP**) which initiates a set of responses leading to a lowering of blood pressure.

The Vagus Nerves

The vagus nerves are part of the parasympathetic branch of the **autonomic nervous system**. They, too, run from the medulla oblongata to the heart. Their activity **slows** the heartbeat. Pressure receptors in the aorta and carotid arteries send impulses to the medulla which relays these by way of the vagus nerves to the heart. Heartbeat and blood pressure diminish.

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15.3D: The Lymphatic System

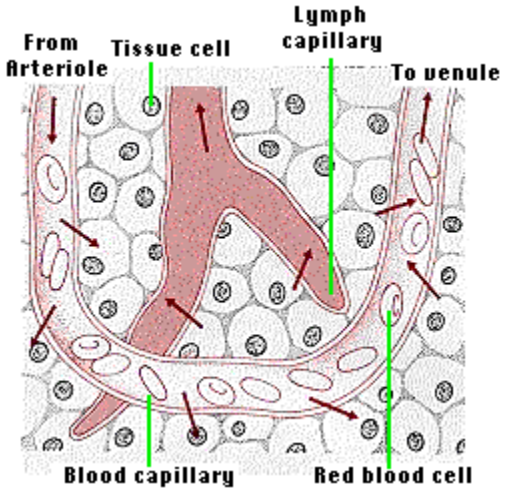


Figure 15.3.4.1 Tissue Space

Most (~90%) of this interstitial fluid returns at the venule end of the capillary. The 10% that does not is picked up by tiny vessels called **lymph capillaries**. The cells forming the walls of the lymph capillaries are loosely fitted together, thus making the wall very porous. Any serum proteins that filtered through the capillary wall pass easily from the tissue space into the interior of the lymph capillary. The lymph capillaries of the intestinal villi, called lacteals, also pick up fat droplets. White blood cells (leukocytes) migrate from the tissue space into the lymph capillary squeezing between the cells that make up its wall.

The lymph capillaries drain into still larger collecting vessels. The flow through the collecting vessels is quite slow. Like blood in the veins, contraction of skeletal muscles compresses the collecting vessels and squeezes the fluid — now called lymph — along. Again, like the return of blood in the veins, the lymph can flow only in one direction because of valves in the vessels. All the lymph collected from the entire left side of the body, the digestive tract and the right side of the lower part of the body flows into a single major vessel, the thoracic duct.

The thoracic duct empties into the left subclavian vein. The lymph in the right side of the head, neck, and chest is collected by the right lymph duct and empties into the right subclavian vein. Together they empty 1–2 liters of lymph into the blood each day.

Lymph Nodes

The collecting vessels of the lymphatic system are interrupted by lymph nodes. These are especially abundant in the groin, armpits, abdomen and neck. These contain cavities called sinuses into which the lymph flows bringing various leukocytes (e.g., lymphocytes and dendritic cells) and out of which pass antibodies and lymphocytes which then enter the blood at the subclavian veins.

The walls of the sinuses are lined with phagocytic cells, which engulf any foreign particles, e.g., bacteria, that might be present in the lymph. Tests have demonstrated that over 99% of the bacteria carried into a node are screened out before the lymph leaves the node on its return to the blood. This filtering mechanism is one of the most important body defenses against infectious disease. When combating a heavy infection, the lymph nodes enlarge producing "swollen glands".

Edema

The production of lymph is increased by

- increased blood pressure in the capillaries
- increased capillary permeability such as occurs if the adherens junctions between the cells lining the capillaries are damaged
- a decreased concentration of plasma proteins (such as occurs in prolonged malnutrition).

The lymphatic system may be unable to handle the increased volume of lymph, and it may accumulate in the tissues and distend them. This condition is known as edema.

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15.3E: Blood

Blood is a liquid tissue. Suspended in the watery plasma are seven types of cells and cell fragments.

1. red blood cells (RBCs) or erythrocytes
2. platelets or thrombocytes
3. five kinds of white blood cells (WBCs) or leukocytes
    - Three kinds of granulocytes
        - neutrophils
        - eosinophils
        - basophils
    - Two kinds of leukocytes without granules in their cytoplasm
        - lymphocytes
        - monocytes

If one takes a sample of blood, treats it with an agent to prevent clotting, and spins it in a centrifuge, the red cells settle to the bottom and the white cells settle on top of them forming the "buffy coat". The fraction occupied by the red cells is called the hematocrit. Normally it is approximately 45%. Values much lower than this are a sign of anemia

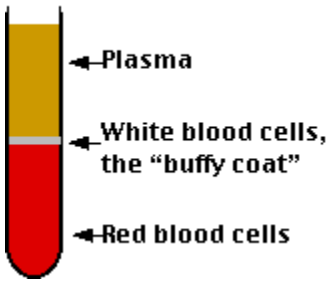


Figure 15.3.5.1 Hematocrit

Functions of the blood

Blood performs two major functions:

- transport through the body of
    - oxygen and carbon dioxide
    - food molecules (glucose, lipids, amino acids)
    - ions (e.g., Na^{+}, Ca^{2+}, HCO_3^{-})
    - wastes (e.g., urea)
    - hormones
    - heat
- defense of the body against infections and other foreign materials. All the WBCs participate in these defenses.

The formation of blood cells

All the various types of blood cells are produced in the bone marrow (some 10^{11} of them each day in an adult human). They arise from a single type of cell called a **hematopoietic stem cell** — an "adult" multipotent stem cell. These stem cells are very rare (only about one in 10,000 bone marrow cells) and are attached (probably by adherens junctions) to osteoblasts lining the inner surface of bone cavities. They also express a cell-surface protein designated CD34.

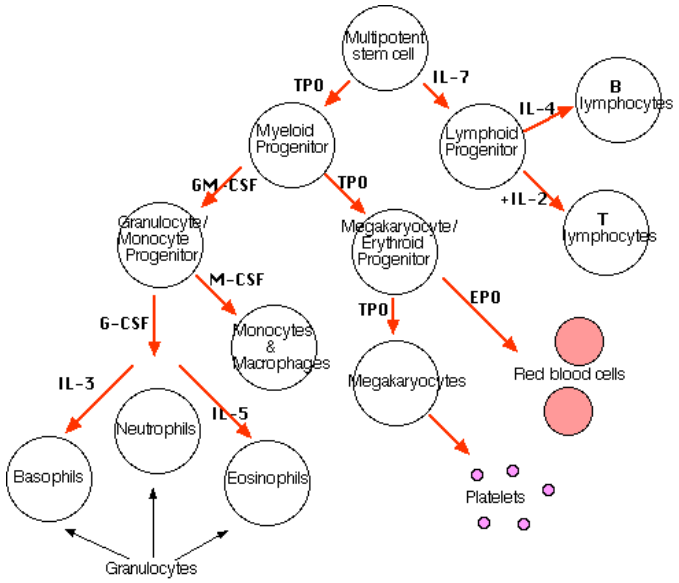


Figure $15.3E.1$: Types of blood cells

Hematopoietic stem cell produce, by mitosis, two kinds of progeny: (1) more stem cells (A mouse that has had all its blood stem cells killed by a lethal dose of radiation can be saved by the injection of a single living stem cell) and (2) cells that begin to differentiate along the paths leading to the various kinds of blood cells. Which path is taken is regulated by the need for more of that type of blood cell which is, in turn, controlled by appropriate cytokines and/or hormones. For example, Interleukin-7 (IL-7) is the major cytokine in stimulating bone marrow stem cells to start down the "lymphoid" path leading to the various lymphocytes (mostly B cells and T cells).

Some of the cytokines that drive the differentiation of the "myeloid" leukocytes are

- **Erythropoietin** (EPO), produced by the kidneys, enhances the production of red blood cells (RBCs).
- **Thrombopoietin** (TPO), assisted by Interleukin-11 (IL-11), stimulates the production of megakaryocytes. Their fragmentation produces platelets.
- **Granulocyte-macrophage colony-stimulating factor (GM-CSF)**, as its name suggests, sends cells down the path leading to both those cell types. In due course, one path or the other is taken.
  - Under the influence of granulocyte colony-stimulating factor (G-CSF), they differentiate into neutrophils.
  - Further stimulated by interleukin-5 (IL-5) they develop into eosinophils.
  - Interleukin-3 (IL-3) participates in the differentiation of most of the white blood cells but plays a particularly prominent role in the formation of basophils (responsible for some allergies).
  - Stimulated by macrophage colony-stimulating factor (M-CSF) the granulocyte/macrophage progenitor cells differentiate into monocytes, macrophages, and dendritic cells (DCs).

Red Blood Cells (erythrocytes)

The most numerous type in the blood. They average 7 µm in diameter. Women average about 4.8 million of these cells per cubic millimeter (mm^3; which is the same as a microliter [µl]) of blood. Men average about 5.4 x 10^6 per µl. These values can vary over quite a range depending on such factors as health and altitude. (Peruvians living at 18,000 feet may have as many as 8.3 x 10^6 RBCs per µl.)

RBC precursors mature in the bone marrow closely attached to a macrophage. They manufacture hemoglobin until it accounts for some 90% of the dry weight of the cell. In mammals, the nucleus is squeezed out of the cell and is ingested by the macrophage. All the mitochondria as well as the endoplasmic reticulum and Golgi apparatus are destroyed. No-longer-needed proteins are expelled from the cell in vesicles called exosomes. Figure $15.3E.2$ shows a scanning electron micrograph that shows the characteristic biconcave shape of red blood cells.

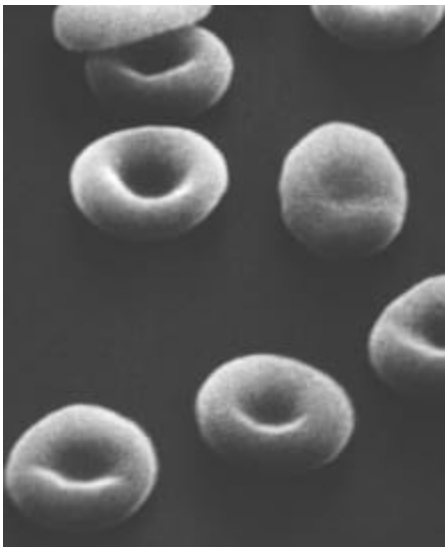


Figure $15.3E.2$: Red blood cells courtesy of Dr. Marion J. Barnhart

Thus RBCs are terminally differentiated; that is, they can never divide. They live about 120 days and then are ingested by phagocytic cells in the liver and spleen. Most of the iron in their hemoglobin is reclaimed for reuse. The remainder of the heme portion of the molecule is degraded into bile pigments and excreted by the liver. Some 3 million RBCs die and are scavenged by the liver each second.

Red blood cells are responsible for the transport of oxygen and carbon dioxide.

Oxygen Transport

In adult humans the hemoglobin (Hb) molecule consists of four polypeptides:

- two alpha (α) chains of 141 amino acids
- two beta (β) chains of 146 amino acids

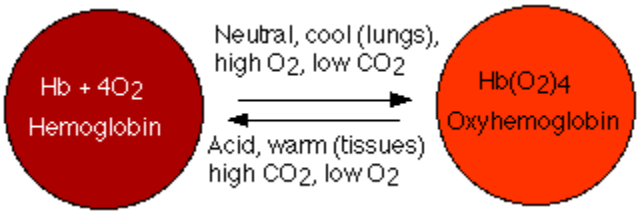


Figure $15.3E.3$: oxyhemoglobin

To each of these is attached the prosthetic group heme. There is one atom of iron at the center of each heme. One molecule of oxygen can bind to each heme. The reaction is reversible. Under the conditions of lower temperature, higher pH, and increased oxygen pressure in the capillaries of the lungs, the reaction proceeds to the right. The purple-red deoxygenated hemoglobin of the venous blood becomes the bright-red oxyhemoglobin of the arterial blood. Under the conditions of higher temperature, lower pH, and lower oxygen pressure in the tissues, the reverse reaction is promoted and oxyhemoglobin gives up its oxygen.

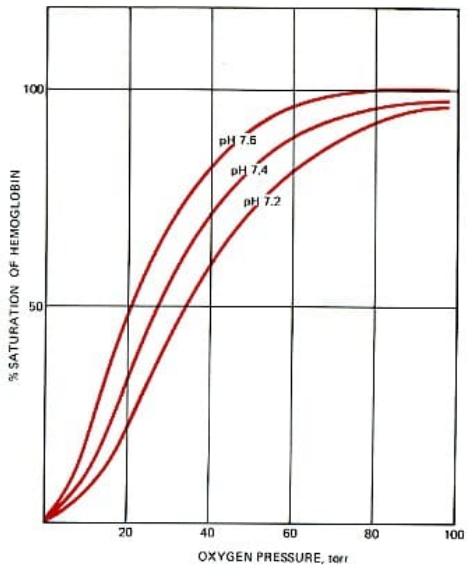


Figure $15.3E.4$: Oxygen pressure vs hemoglobin saturation

The pressure of oxygen in the lungs is 90–95 torr; in the interior tissues it is about 40 torr. Therefore, only a portion of the oxygen carried by the red blood cells is normally unloaded in the tissues. However, vigorous activity can lower the oxygen pressure in skeletal muscles below 40 torr, which causes a large increase in the amount of oxygen released. This effect is enhanced by the high concentration of carbon dioxide in the muscles and the resulting lower pH (7.2). The lower carbon dioxide concentration (and hence higher pH) at the lungs promotes the binding of oxygen to hemoglobin and hence the uptake of oxygen.

Temperature changes also influence the binding of oxygen to hemoglobin. In the relative warmth of the interior organs, the curve is shifted to the right (like the curve for pH 7.2), helping to unload oxygen. In the relative coolness of the lungs, the curve is shifted to the left, aiding the uptake of oxygen.

Although the oxygen transported by RBCs make possible cellular respiration throughout the body, RBCs lack mitochondria and so cannot perform cellular respiration themselves and must subsist on glycolysis.

Carbon Dioxide Transport

Carbon dioxide (CO_2) combines with water forming carbonic acid, which dissociates into a hydrogen ion (H^+) and a bicarbonate ion:

$$\mathrm{CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-} \tag{15.3E.1}$$

95% of the CO_2 generated in the tissues is carried in the red blood cells. It enters (and leaves) the cell by diffusion through the plasma membrane. Once inside, about one-half of the CO_2 is directly bound to hemoglobin (at a site different from the one that binds oxygen). The rest is converted following the equation above by the enzyme carbonic anhydrase into bicarbonate ions that diffuse back out into the plasma and hydrogen ions (H^+) that bind to the protein portion of the hemoglobin (thus having no effect on pH). The bicarbonate ions pass out of the red cell by facilitated diffusion through transmembrane channels in the plasma membrane. Only about 5% of the CO_2 generated in the tissues dissolves directly in the plasma. (A good thing, too: if all the CO_2 we make were carried this way, the pH of the blood would drop from its normal 7.4 to an instantly-fatal 4.5) When the red cells reach the lungs, these reactions are reversed and CO_2 is released to the air of the alveoli.

Anemia

Anemia is a shortage of RBCs and/or the amount of hemoglobin in them. Anemia has many causes. One of the most common is an inadequate intake of iron in the diet.

Blood Groups

Red blood cells have surface antigens that differ between people and that create the so-called blood groups such as the ABO system and the Rh system.

White Blood Cells (leukocytes)

White blood cells are much less numerous than red (the ratio between the two is around 1:700) and have nuclei. They consist of lymphocytes and monocytes with relatively clear cytoplasm, and three types of granulocytes, whose cytoplasm is filled with granules. They participate in protecting the body from infection.

Lymphocytes

There are several kinds of lymphocytes (although they all look alike under the microscope), each with different functions to perform . The most common types of lymphocytes are:

- B lymphocytes ("B cells"). These are responsible for making antibodies.
- T lymphocytes ("T cells"). There are several subsets of these:
  - inflammatory T cells that recruit macrophages and neutrophils to the site of infection or other tissue damage
  - cytotoxic T lymphocytes (CTLs) that kill virus-infected and, perhaps, tumor cells
  - helper T cells that enhance the production of antibodies by B cells

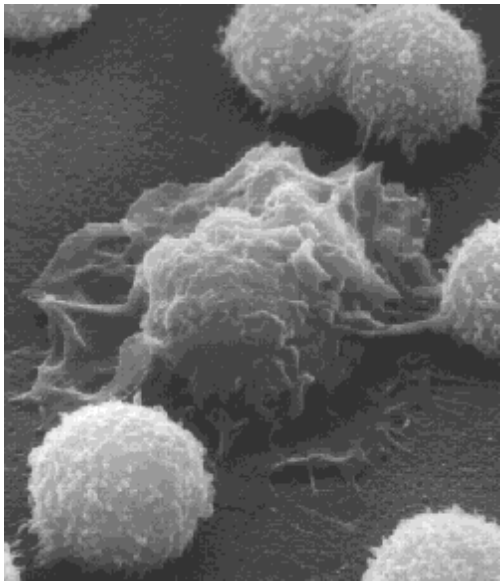


Figure 15.3.5.6 Lymphocytes surrounding a macrophage courtesy of Drs. Jan M. Orenstein and Emma Shelton

Although bone marrow is the ultimate source of lymphocytes, the lymphocytes that will become T cells migrate from the bone marrow to the thymus where they mature. Both B cells and T cells also take up residence in lymph nodes, the spleen and other tissues where they encounter antigens, continue to divide by mitosis, mature into fully functional cells.

Monocytes

Monocytes leave the blood and become macrophages and one type of dendritic cell. See Figure 15.3.5.6

Neutrophils

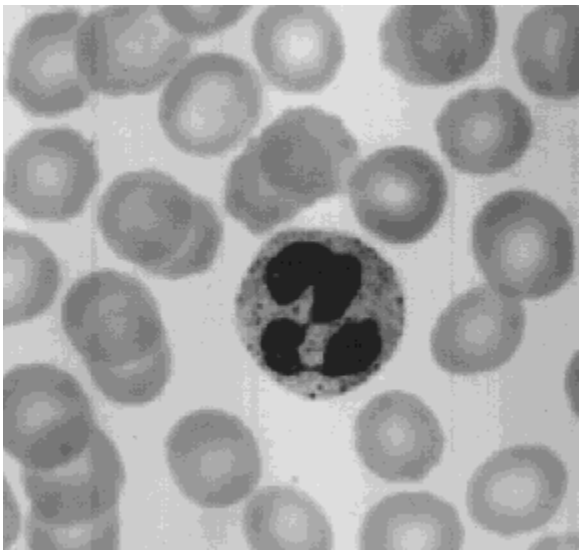


Figure 15.3.5.7 Neutorphils

The most abundant of the WBCs. This photomicrograph shows a single neutrophil surrounded by red blood cells. Neutrophils squeeze through the capillary walls and into infected tissue where they kill the invaders (e.g., bacteria) and then engulf the remnants by phagocytosis. This is a never-ending task, even in healthy people: Our throat, nasal passages, and colon harbor vast numbers of bacteria. Most of these are commensals, and do us no harm. But that is because neutrophils keep them in check.

However heavy doses of radiation, chemotherapy and many other forms of stress can reduce the numbers of neutrophils so that formerly harmless bacteria begin to proliferate. The resulting opportunistic infection can be life-threatening.

Eosinophils

The number of eosinophils in the blood is normally quite low (0–450/µl). However, their numbers increase sharply in certain diseases, especially infections by parasitic worms. Eosinophils are cytotoxic, releasing the contents of their granules on the invader.

Basophils

Ordinarily representing less than 1% of the WBCs, their numbers also increase during infection. Basophils leave the blood and accumulate at the site of infection or other inflammation. There they discharge the contents of their granules, releasing a variety of mediators such as histamine, serotonin, prostaglandins and leukotrienes which increase the blood flow to the area and in other ways add to the inflammatory process. The mediators released by basophils also play an important part in some allergic responses such as hay fever and an anaphylactic response to insect stings.

Platelets

Platelets are cell fragments produced from megakaryocytes. These polyploid (128n) cells in the bone marrow send pseudopodia-like projections into the lumen of adjacent blood vessels. Blood flowing through the vessel shears off the platelets. Blood normally contains 150,000–400,000 per microliter (µl) or cubic millimeter (mm^3). This number is normally maintained by a homeostatic (negative-feedback) mechanism. If this value should drop much below 20,000/µl, there is a danger of uncontrolled bleeding. Some causes include certain drugs and herbal remedies as well as autoimmunity.

When blood vessels are cut or damaged, the loss of blood from the system must be stopped before shock and possible death occur. This is accomplished by solidification of the blood, a process called coagulation or clotting. A blood clot consists of a plug of platelets enmeshed in a network of insoluble fibrin molecules. Platelets also promote inflammation.

Plasma

Plasma is the straw-colored liquid in which the blood cells are suspended.

Composition of blood plasma

Component	Percent
Water	~92
Proteins	6–8
Salts	0.8
Lipids	0.6
Glucose (blood sugar)	0.1

Plasma transports materials needed by cells and materials that must be removed from cells:

- various ions (Na^+, Ca^{2+}, HCO_3^-, etc.)
- glucose and traces of other sugars
- amino acids
- other organic acids
- cholesterol and other lipids
- hormones
- urea and other wastes

Most of these materials are in transit from a place where they are added to the blood (a "source")

- exchange organs like the intestine
- depots of materials like the liver

to places ("sinks") where they will be removed from the blood.

- every cell
- exchange organs like the kidney, and skin.

Serum Proteins

Proteins make up 6–8% of the blood. They are about equally divided between serum albumin and a great variety of serum globulins. After blood is withdrawn from a vein and allowed to clot, the clot slowly shrinks. As it does so, a clear fluid called serum is squeezed out. Thus Serum is blood plasma without fibrinogen and other clotting factors. The serum proteins can be separated by electrophoresis.

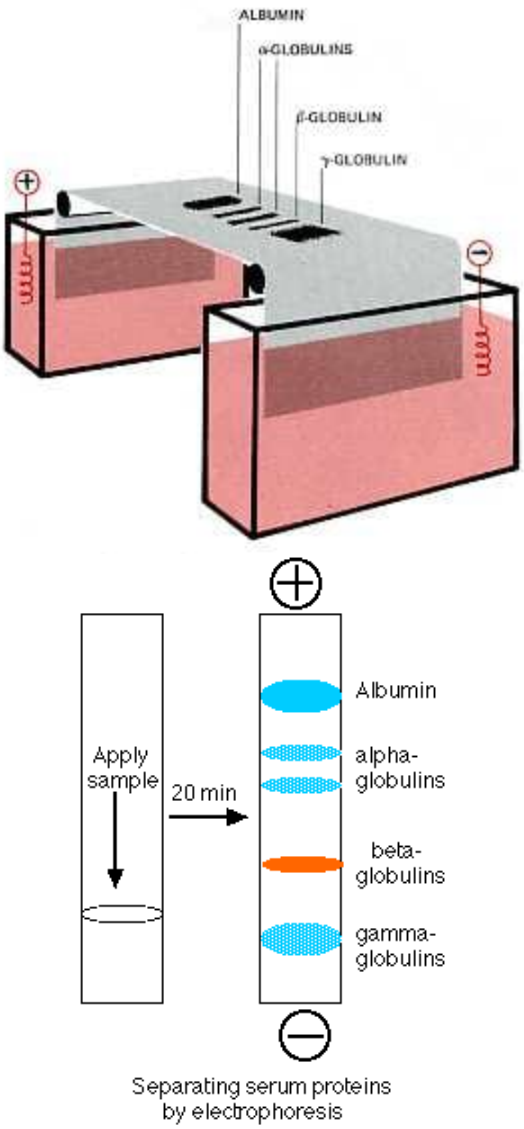


Figure 15.3.5.8 Electrophoresis

- A drop of serum is applied in a band to a thin sheet of supporting material, like paper, that has been soaked in a slightly-alkaline salt solution.
- At pH 8.6, which is commonly used, all the proteins are negatively charged, but some more strongly than others.
- A direct current can flow through the paper because of the conductivity of the buffer with which it is moistened.
- As the current flows, the serum proteins move toward the positive electrode.
- The stronger the negative charge on a protein, the faster it migrates.
- After a time (typically 20 min), the current is turned off and the proteins stained to make them visible (most are otherwise colorless).
- The separated proteins appear as distinct bands.
- The most prominent of these and the one that moves closest to the positive electrode is serum albumin.
- Serum albumin
  - is made in the liver
  - binds many small molecules for transport through the blood
  - helps maintain the osmotic pressure of the blood
- The other proteins are the various serum globulins.
- They migrate in the order
  - alpha globulins (e.g., the proteins that transport thyroxine and retinol [vitamin A])
  - beta globulins (e.g., the iron-transporting protein transferrin)
  - gamma globulins.

- Gamma globulins are the least negatively-charged serum proteins. (They are so weakly charged, in fact, that some are swept in the flow of buffer back toward the negative electrode.)
- Most antibodies are gamma globulins.
- Therefore gamma globulins become more abundant following infections or immunizations.
- Gamma globulins can be harvested from donated blood (usually pooled from several thousand donors) and injected into persons exposed to certain diseases such as chicken pox and hepatitis. Because such preparations of immune globulin contain antibodies against most common infectious diseases, the patient gains temporary protection against the disease.

If a precursor of an antibody-secreting cell becomes cancerous, it divides uncontrollably to generate a clone of plasma cells secreting a single kind of antibody molecule.

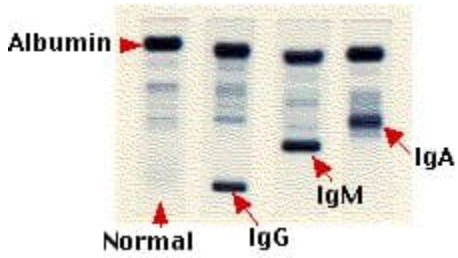


Figure 15.3.5.8 Electrophoretic seperation courtesy Beckman Instruments, Inc.

The above image shows — from left to right — the electrophoretic separation of:

- normal human serum with its diffuse band of gamma globulins
- serum from a patient with multiple myeloma producing an IgG myeloma protein
- serum from a patient with Waldenström's macroglobulinemia where the cancerous clone secretes an IgM antibody
- serum with an IgA myeloma protein

Serum Lipids

Because of their relationship to cardiovascular disease, the analysis of serum lipids has become an important health measure. The table shows the range of typical values as well as the values above (or below) which the subject may be at increased risk of developing atherosclerosis.

LIPID	Typical values (mg/dl)	Desirable (mg/dl)
Cholesterol (total)	170–210	<200
LDL cholesterol	60–140	<100
HDL cholesterol	35–85	>40
Triglycerides	40–160	<160

- Total cholesterol is the sum of HDL cholesterol, LDL cholesterol and 20% of the triglyceride value
- Note that high LDL values are bad, but high HDL values are good.
- Using the various values, one can calculate a
  cardiac risk ratio = total cholesterol divided by HDL cholesterol
- A cardiac risk ratio greater than 7 is considered a warning.

Blood Transfusions

In the United States, in 2001, some 15 million "units" (~475 ml) of blood were collected from blood donors.

- Some of these units ("whole blood") were transfused directly into patients (e.g., to replace blood lost by trauma or during surgery).
- Most were further fractionated into components, including:

- RBCs - When refrigerated these can be used for up to 42 days.
- Platelets - These must be stored at room temperature and thus can be saved for only 5 days.
- Plasma - This can be frozen and stored for up to a year.

Ensuring the safety of donated blood

A variety of infectious agents can be present in blood. Infections such as

- viruses (e.g., HIV-1, hepatitis B and C, West Nile virus)
- bacteria like the spirochete of syphilis
- protozoans like the agents of malaria and babesiosis
- prions (e.g., the agent of variant Crueutzfeldt-Jakob disease)

These could be transmitted to recipients. To minimize these risks donors are questioned about their possible exposure to these agents. Each unit of blood is tested for a variety of infectious agents. Most of these tests are performed with enzyme immunoassays (EIA) and detect antibodies against the agents. However, it takes a period of time for the immune system to produce antibodies following infection, and during this period ("window"), infectious virus is present in the blood. For this reason, blood is now also checked for the presence of the RNA of these RNA viruses HIV-1, hepatitis C and West Nile virus by the nucleic acid-amplification test (NAT).

Thanks to all these precautions, the risk of acquiring an infection from any of these agents is vanishingly small. Despite this, some people — in anticipation of need — donate their own blood ("autologous blood donation") prior to surgery.

Blood Typing

Donated blood must also be tested for certain cell-surface antigens that might cause a dangerous transfusion reaction in an improperly-matched recipient.

Blood Substitutes

Years of research have gone into trying to avoid the problems of blood perishability and safety by developing blood substitutes. Most of these have focused on materials that will transport adequate amounts of oxygen to the tissues.

- Some are totally synthetic substances.
- Others are derivatives of hemoglobin.

Although some have reached clinical testing, none has as yet proved acceptable for routine use.

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15.3F: Blood Groups

Blood groups are created by molecules present on the surface of red blood cells (and often on other cells as well).

The ABO Blood Groups

The **ABO blood groups** were the first to be discovered (in 1900) and are the most important in assuring safe blood transfusions. The table shows the four ABO phenotypes ("blood groups") present in the human population and the genotypes that give rise to them.

Blood Group	Antigens on RBCs	Antibodies in Serum	Genotypes
A	**A**	Anti-B	*AA* or *AO*
B	**B**	Anti-A	*BB* or *BO*
AB	**A** and **B**	Neither	*AB*
O	Neither	Anti-A and Anti-B	*OO*

When red blood cells carrying one or both antigens are exposed to the corresponding antibodies, they agglutinate; that is, clump together. People usually have antibodies against those red cell antigens that they lack. The antigens in the ABO system are O-linked glycoproteins with their sugar residues exposed at the cell surface. The terminal sugar determines whether the antigen is A or B.

The critical principle to be followed is that transfused blood must not contain red cells that the **recipient's** antibodies can clump. Although theoretically it is possible to transfuse group O blood into any recipient, the antibodies in the donated plasma can damage the recipient's red cells. Thus, when possible, transfusions should be done with exactly-matched blood.

In 2007, Danish and French investigators reported the properties of two bacterial glycosidases that specifically remove the sugars responsible for the A and B antigens. This discovery raises the possibility of being able to treat A, B, or AB blood with these enzymes and thus convert the blood to Group O, the "universal donor".

Why do we have antibodies against red cell antigens that we lack? Bacteria living in our intestine, and probably some foods, express epitopes similar to those on A and B. We synthesize antibodies against these if we do not have the corresponding epitopes; that is, if our immune system sees them as "foreign" rather than "self".

The Rh System

Rh antigens are transmembrane proteins with loops exposed at the surface of red blood cells. They appear to be used for the transport of carbon dioxide and/or ammonia across the plasma membrane. They are named for the rhesus monkey in which they were first discovered.

There are a number of Rh antigens. Red cells that are "Rh-positive" express the one designated **D**. About 15% of the population have no RhD antigen and thus are "Rh-negative". The major importance of the Rh system for human health is to avoid the danger of RhD incompatibility between mother and fetus.

During birth, there is often a leakage of the baby's red blood cells into the mother's circulation. If the baby is Rh-positive (having inherited the trait from its father) and the mother Rh-negative, these red cells will cause her to develop antibodies against the RhD antigen. The antibodies, usually of the IgG class, do not cause any problems for that child, but can cross the placenta and attack the red cells of a subsequent Rh^+ fetus. This destroys the red cells producing anemia and jaundice. The disease, called **erythroblastosis fetalis** or **hemolytic disease of the newborn**, may be so severe as to kill the fetus or even the newborn infant. It is an example of an antibody-mediated cytotoxicity disorder.

Although certain other red cell antigens (in addition to Rh) sometimes cause problems for a fetus, an **ABO** incompatibility does

not. Why is an Rh incompatibility so dangerous when ABO incompatibility is not?

It turns out that most anti-A or anti-B antibodies are of the IgM class and these do **not** cross the placenta. In fact, an **Rh^-/type O** mother carrying an **Rh^+/type A, B, or AB** fetus is resistant to sensitization to the Rh antigen. Presumably her anti-A and anti-B antibodies destroy any fetal cells that enter her blood before they can elicit anti-Rh antibodies in her. This phenomenon has led to an extremely effective preventive measure to avoid Rh sensitization. Shortly after each birth of an Rh^+ baby, the mother is given an injection of anti-Rh antibodies. The preparation is called **Rh immune globulin** (**RhIG**) or **Rhogam**. These passively acquired antibodies destroy any fetal cells that got into her circulation before they can elicit an active immune response in her.

Rh immune globulin came into common use in the United States in 1968, and within a decade the incidence of Rh hemolytic disease became very low.

Other blood groups

Several other blood group antigens have been identified in humans. Some examples: **MN**, Duffy, Lewis, Kell.

These groups also sometimes cause transfusion reactions and even hemolytic disease of the newborn in cases where there is no ABO or Rh incompatibility. The Duffy red cell antigen also serves as the receptor for entry by the malaria parasite Plasmodium vivax.

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15.3G: The Transport of Heat

In addition to its role in the transport of materials, the circulatory system is responsible for the distribution of heat throughout the body. This is true both of

- **endotherms**, animals — birds and mammals — that generate **internally** the heat needed to maintain their body temperature. Birds and mammals are "warm-blooded' or **homeothermic**, maintaining their body temperature within narrow limits, no matter what the ambient temperature.
- **mesotherms**, animals that generate heat internally but do not maintain a fixed body temperature. Tuna, some sharks, and the echidna are mesotherms.
- **ectotherms**, animals — the other vertebrates and the invertebrates — that secure their heat from their surroundings (e.g., by basking in the sun). Ectotherms are "cold-blooded" or **poikilothermic**.

The major source of heat for endotherms is the metabolism of their internal organs. Over two-thirds of the heat generated in a resting human is created by the organs of the thoracic and abdominal cavities and the brain (which contributes 16% of the total — about the same as all our skeletal muscles when they are at rest). There are several measures that an endothermic animal can take if it begins to lose heat to its surroundings faster than it can generate heat (i.e., it begins to grow cold). It can increase the metabolic rate of its tissues. Many small mammals and human infants do this as their surroundings get colder, but it is still uncertain whether adult humans can. The increase in metabolism, with the accompanying release of heat, occurs in brown adipose tissue. It can also increase its physical activity. At rest, muscles make only a small contribution (about 16%) to body heat. During vigorous exercise, this can increase greatly. In the absence of voluntary muscle action, the same effect is achieved by **shivering**. The greater the surface-to-volume ratio of a part of the body, the faster is can transfer heat to its surroundings. This is why you first notice cold in your hands and feet. The loss of heat from the extremities can be sharply reduced by diminishing their blood supply. In extreme cold, for example, the blood supply to the fingers can drop to 1% or so of its normal value.

Countercurrent heat exchanger

Many animals (including humans) have another way to conserve heat. The arteries of our arms and legs run parallel to a set of deep veins. As warm blood passes down the arteries, the blood gives up some of its heat to the colder blood returning from the extremities in these veins. Such a mechanism is called a **countercurrent heat exchanger**. When heat loss is no problem, most of the venous blood from the extremities returns through veins located near the surface.

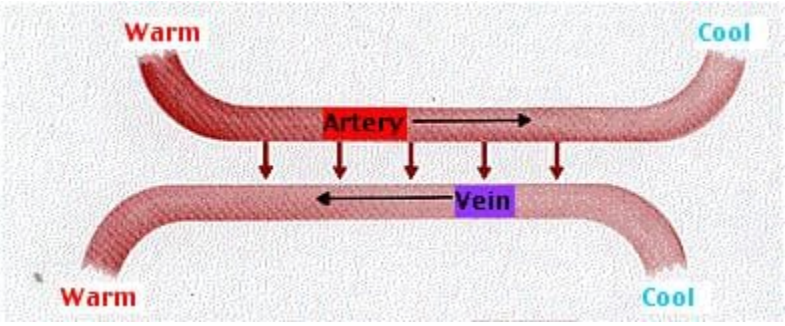


Figure 15.3.7.1 Countercurrent heat exchanger

Countercurrent heat exchangers can operate with remarkable efficiency. A sea gull can maintain a normal temperature in its torso while standing with its unprotected feet in freezing water. When you consider that the blood of fishes passes over the gills which are bathed in the surrounding water, it is easy to see why fishes are "cold-blooded". Nonetheless, some marine fishes (e.g., the tuna) are mesotherms — able to keep their most active swimming muscles warmer than the sea by using a countercurrent heat exchanger.

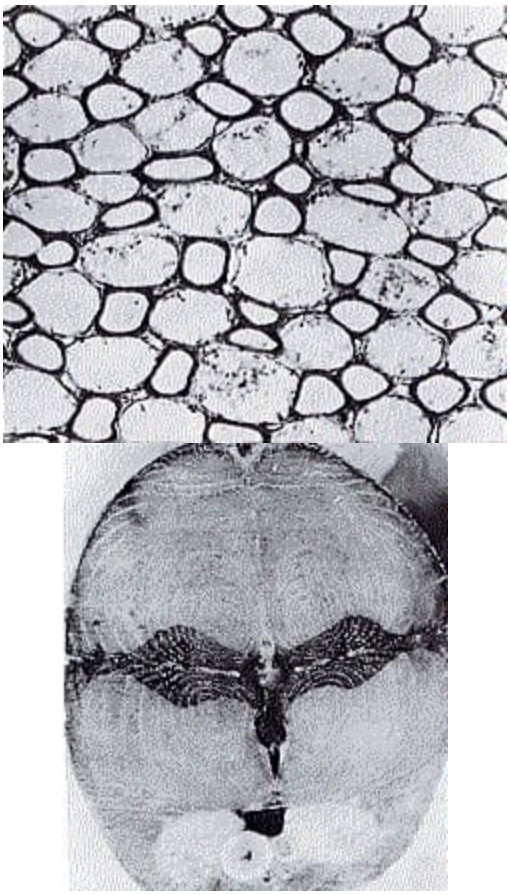


Figure 15.3.7.2 Skipjack courtesy of E. D. Stevens, Dept. of Zoology, University of Guelph, Ontario

The above photograph on the right shows a cross section through a skipjack tuna. The dark muscle on either side of the vertebral column is maintained at a higher temperature than the rest of the fish thanks to its countercurrent heat exchanger. The cold, oxygen-rich arterial blood passes into a series of fine arteries that take the blood into the active muscles. These fine arteries lie side by side with veins draining those muscles. So as the cold blood passes into the muscles, it picks up the heat that had been generated by these muscles and keeps it from being lost to the surroundings. Thanks to this countercurrent heat exchanger, a tuna swimming in the winter can maintain its active swimming muscles 14°C warmer than the surrounding water. The photomicrograph on the left is of a cross section through the heat exchanger. Note the close, parallel packing of the arteries (thick walls) and veins (thin walls).

Countercurrent exchangers also operate in the kidney and are built into the design of artificial kidneys.

The circulatory system is also responsible for cooling an animal. If the animal's "core" body temperature gets too high, the blood supply to the surface and extremities is increased enabling heat to be released to the surroundings. If this is insufficient, the animal can evaporate water from the blood — in the form of sweat for those animals with sweat glands. The evaporation of 1 gram of water absorbs some 540 calories of heat.

Most endotherms cannot tolerate a rise in body temperature of more than 5°C or so. The brain is the organ most susceptible to damage by a high temperature. Some mammals, dogs for example, have a countercurrent heat exchanger located between the carotid arteries and the vessels that distribute blood to the brain. This heat exchanger transfers some of the heat of the arterial blood to the relatively cool venous blood returning from the nose and mouth. This cools their arterial blood before it reaches the brain.

The shifting of blood flow as needed to maintain homeothermy is controlled by temperature receptors in the hypothalamus of the brain. One set of receptors here responds to small (0.01°C) increases in the temperature of the blood. When triggered, all the activities such as shunting blood vessels to the skin and extremities and sweating by which the body cools itself are brought into play. It is this center that enables us to maintain a constant body temperature (homeothermy) during periods of extreme exertion or in hot surroundings.

A second region of the hypothalamus triggers warming responses such as shunting blood away from the skin and extremities and shivering when the body becomes chilled. It is the hypothalamus that executes the **fever** response. In effect, the hypothalamus is the body's thermostat. The release of prostaglandins during inflammation increases the setting; that is, turns the thermostat "up". If the body temperature is not yet there, the body begins shivering violently — causing "chills" — to generate the heat needed. The result is fever when the new set point is reached.

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15.3H: Blood Clotting

When blood vessels are cut or damaged, the loss of blood from the system must be stopped before shock and possible death occur. This is accomplished by solidification of the blood, a process called *coagulation* or *clotting*. A blood clot consists of a plug of platelets enmeshed in a network of insoluble fibrin molecules. Platelet aggregation and fibrin formation both require the proteolytic enzyme thrombin. Clotting also requires calcium ions (Ca^{2+}) (which is why blood banks use a chelating agent to bind the calcium in donated blood so the blood will not clot in the bag) and about a dozen other protein clotting factors. Most of these circulate in the blood as inactive precursors. They are activated by proteolytic cleavage becoming, in turn, active proteases for other factors in the system. By tradition, these factors are designated by Roman numerals.

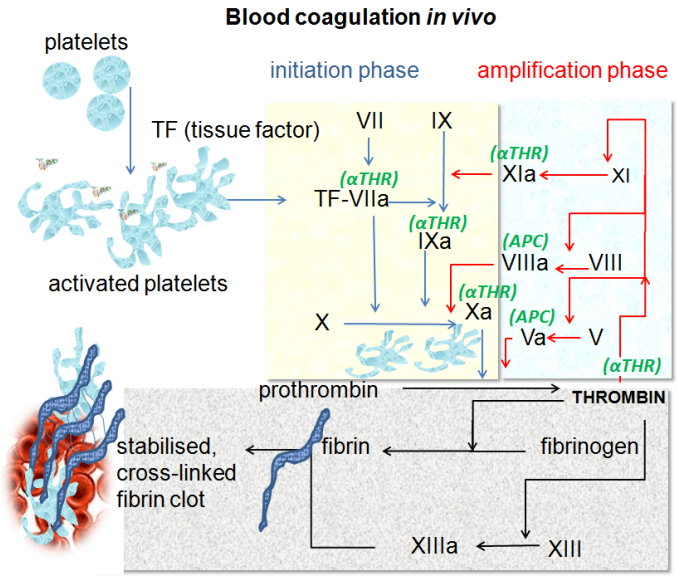


Figure 15.3.8.1: Clotting process. Blood coagulation pathways in vivo showing the central role played by thrombin. Image used wtih permission (CC BY-SA 3.0; Dr Graham Beards).

There are two processes that can initiate clotting: A very rapid process the so-called **extrinsic** pathway or a slower but larger **intrinsic** pathway.

The Extrinsic Pathway

Damaged cells display a surface protein called **tissue factor** (**TF**). Tissue factor then binds to activated Factor **7.** The **TF-7** heterodimer is a protease with two substrates: Factor **9** and Factor **10.**

The Intrinsic Pathway

Factor **12** (also called the Hageman factor) circulates in the blood. If blood escapes into tissue spaces (e.g., as a result of an injury), contact with collagens in the tissue space activates Factor 12. Activated Factor 12 is a serine protease that activates Factor **11** which in turn activates Factor **9** which in turn activates Factor **10**.

The Two Pathways Converge at Factor 10

Factor **10 -** produced by either or, more likely, both pathways - binds and activates Factor **5**. This heterodimer is called **prothrombinase** because it is a protease that converts **prothrombin** (also known as Factor II) to **thrombin**. Thrombin has several different activities. Two of them are:

- proteolytic cleavage of **fibrinogen** (aka "Factor I") to form soluble molecules of **fibrin** and a collection of small and **fibrinopeptides.**

- activation of Factor **13** which forms covalent bonds between the soluble fibrin molecules converting them into an insoluble meshwork — the **clot**.

Thrombin and activated Factors 10 ("Xa") and 11 ("XIa") are also **serine proteases**.

Amplifying the Clotting Process

The clotting process also has several **positive feedback loops** which quickly magnify a tiny initial event into what may well be a lifesaving plug to stop bleeding.

- The TF-7 complex (which started the process) also activates Factor **9**.
  - Factor 9 binds to Factor **8**, a protein that circulates in the blood stabilized by another protein, **von Willebrand Factor** (**vWF**).
  - This complex activates more Factor **10**.
- As thrombin is generated, it activates more
  - Factor **5**
  - Factor **8**
  - Factor **11** (all shown above with green arrows).
- Factor 11 amplifies the production of activated Factor **9**.

Thus what may have begun as a tiny, localized event rapidly expands into a cascade of activity.

Platelets

Platelets are cell fragments produced from **megakaryocytes**. Blood normally contains 150,000 to 400,000 per microliter (µl). If this value should drop much below 20,000/µl, there is a danger of uncontrolled bleeding. This is because of the essential role of platelets in maintaining the integrity of the adherens junctions that provide a tight seal between the endothelial cells that line the blood vessels and in forming a clot where blood vessels have been broken.

When blood vessels are damaged, fibrils of collagen in the extracellular matrix (ECM) are exposed. Platelets then begin to adhere to the collagen through the action of specific receptors for collagen present on their plasma membrane and **von Willebrand factor** which links the platelets to the collagen. These actions cause a plug of platelets to form at the site.

The bound platelets release ADP and **thromboxane A2**, which recruit and activate still more platelets circulating in the blood. (This role of thromboxane accounts for the beneficial effect of low doses of aspirin — a cyclooxygenase inhibitor — in avoiding heart attacks.), tissue factor, and serotonin, which enhances their clumping and promotes constriction of the blood vessel.

ReoPro

ReoPro s a monoclonal antibody directed against platelet receptors. It inhibits platelet aggregation and appears to reduce the risk that "reamed out" coronary arteries (after coronary angioplasty) will plug up again.

Bleeding Disorders

A deficiency of a clotting factor can lead to uncontrolled bleeding. The deficiency may arise because not enough of the factor is produced or a mutant version of the factor fails to perform properly.

Examples:

- **von Willebrand disease** (the most common)
- **hemophilia A** for **factor 8** deficiency
- **hemophilia B** for **factor 9** deficiency.
- **hemophilia C** for factor **11** deficiency

In some cases of von Willebrand disease, either a **deficient level** or a **mutant version** of the factor eliminates its protective

effect on factor 8. The resulting low level of factor 8 mimics hemophilia A.

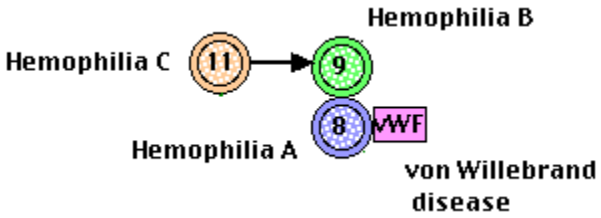


Figure 15.3.8.2 Bleeding disorders

Why do all the human bleeding disorders involve factors in amplification pathways? Probably because they are the only deficiencies that can be tolerated. Loss of the genes for **tissue factor** or factor **7** in knockout mice is lethal.

Hemophilia A and B

The genes encoding factors **8** and **9** are on the X chromosome. Thus their inheritance is X-linked. Like other X-linked disorders, hemophilia A and B are found almost exclusively in males because they inherit just a single X chromosome, and if the gene for factor 8 (or 9) on it is defective, they will suffer from the disease.

Queen Victoria of the UK was a carrier of a mutant factor 9 gene and passed it on to several of her descendants.

There are many different mutant versions of the genes for factors **8** and **9**. Although some produce only a minor effect on the function of their protein, others fail to produce any functioning clotting factor.

Treating Hemophilia A and B

What can be done?

Factor **8** and **9** can be extracted from donated blood, usually pooled from several thousand donors, and purified. Injections of this material can halt episodes of bleeding in hemophiliacs and have allowed countless young men to live relatively normal lives. However, in the early 1980s, blood contaminated with the human immunodeficiency virus (**HIV**) was unknowingly used to manufacture preparations of factors 8 and 9. In some areas, 90% or more of the hemophiliacs became infected by these contaminated preparations. Many have since died of **AIDS**. The future now looks brighter because:

- all donated blood is now tested to see if the donor has been infected with HIV (as well as hepatitis B and C)
- plasma-derived preparations of factors 8 and 9 are now treated with heat and/or solvents to destroy any viruses that might be present
- **recombinant factor 8** and **recombinant factor 9** made by genetic engineering are now available

These recombinant factors are made by inserting the DNA encoding the human protein into mammalian cells grown in culture. E. coli cannot be used because these factors are glycoproteins, and E. coli lacks the machinery to attach carbohydrate properly.

And the team that brought us Dolly reported in the 19 December 1997 issue of **Science** that they have succeeded in cloning female sheep transgenic for the human **factor 9** gene. The human gene is coupled to the promoter for the ovine (sheep) milk protein beta-lactoglobulin. When the lambs mature, it is hoped that they will secrete large amounts of human factor 9 in their milk, which can then be purified for human therapy.

Attempts to cure hemophilia by **gene therapy** also look promising. It is difficult to see how even the most worried critics of genetic engineering can fail to approve its potential to save the lives of thousands of hemophiliacs in the years to come.

Liver Transplants

People with liver failure can be cured with a liver transplant. On the rare occasions when the patient has happened to be a hemophiliac (A, B or C), the transplant cured not only the patient's liver disease but cured his hemophilia as well!

Controlling Clotting

While the ability to clot is essential to life, the process must be carefully regulated. Inappropriate clot formation, especially in the brain or lungs, can be life-threatening.

Antithrombin III

As its name suggests, this plasma protein (a serpin) inhibits the formation of thrombin. It does so by binding to and thus **in**activating prothrombin, factor 9 and factor 10. **Heparin** is a mixture of polysaccharides that bind to antithrombin III, inducing an allosteric change that greatly **enhances** its inhibition of thrombin synthesis. Some surgical patients, especially those receiving hip or heart valve replacements, and people at risk of ischemic stroke (clots in the brain), are given heparin.

Protein C

With its many clot-promoting activities, it is probably no accident that thrombin sits at the center of the control mechanism.

- Excess thrombin binds to cell-surface receptors called **thrombomodulin**.
- The resulting complex activates a plasma protein called **Protein C** and its cofactor **Protein S**.
- Together these inhibit further thrombin formation
    - directly — by inactivating Factor **5**
    - indirectly — by inactivating Factor **8**.

Some inherited disorders that predispose to spontaneous clots, especially in the leg veins:

- inherited deficiency of **Protein C** or **Protein S**
- inherited mutation in the **Factor 5** gene producing a protein that no longer responds to the inhibitory effect of Protein C

Recombinant Protein C is now available to treat people threatened with inappropriate clotting, e.g., as a result of widespread infection (sepsis).

Vitamin K

Vitamin K is a cofactor needed for the synthesis (in the liver) of factors **2** (prothrombin), **7**, **9**, and **10** and proteins C and S. A deficiency of Vitamin K predisposes to bleeding. Conversely, blocking the action of vitamin K helps to prevent inappropriate clotting.

Warfarin (Coumadin®) is sometimes prescribed as a "blood thinner" because it is an effective vitamin K antagonist. (Warfarin is also used as a rat poison because it can cause lethal internal bleeding.) Warfarin treatment is tricky because the therapeutic window (neither too much nor too little) is very narrow, and there is substantial variability between people in their response. So treatment requires regular monitoring of clotting time until the proper dosage is established. However, a number of new anti-clotting agents — that work by inhibiting activated factor 10 (Factor Xa) — are being tested and may turn out to be safer and effective alternatives to warfarin.

Dissolving clots

Plasma contains **plasminogen**, which binds to the fibrin molecules in a clot. Nearby healthy cells release **tissue plasminogen activator** (**TPA**), which also binds to fibrin and, as its name suggests, activates plasminogen forming **plasmin**. Plasmin (another serine protease) proceeds to digest fibrin, thus dissolving the clot.

Recombinant human TPA is now produced by recombinant DNA technology. Injected within the first hours after a heart attack, it has saved many lives by dissolving the clot blocking the coronary artery and restoring blood flow before the heart muscle becomes irreversibly damaged. It is also used for people who suffer an **ischemic stroke**; that is, a **clot** in the brain. (It must not, of course, be used for hemorrhagic strokes, that is, a burst blood vessel)

Angiogenesis

Thrombin (as well as factors 7 and 10) promotes healing by stimulating the growth of new blood vessels at the site of damage.

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15.3I: Sickle-Cell Disease

The most common hemoglobin of adult humans is known as **hemoglobin A** (Hb^A). However, other variant forms of hemoglobin are found in some humans. One of these is called **hemoglobin S** (Hb^S). Individuals who manufacture Hb^S exclusively suffer from sickle-cell disease. Sickle-cell disease is quite common in malaria-ridden parts of Africa and Asia and also occurs in African-Americans and others descended from inhabitants of those regions. The disease gets its name from the fact that the patient's red cells become crescent or sickle-shaped while passing through the capillaries, especially in tissues that are metabolizing actively. The distorted cells are very fragile and are apt to rupture long before their normal life span (about 120 days) is over. This causes a severe anemia (giving rise to an alternate name for the disease: **sickle-cell anemia**).

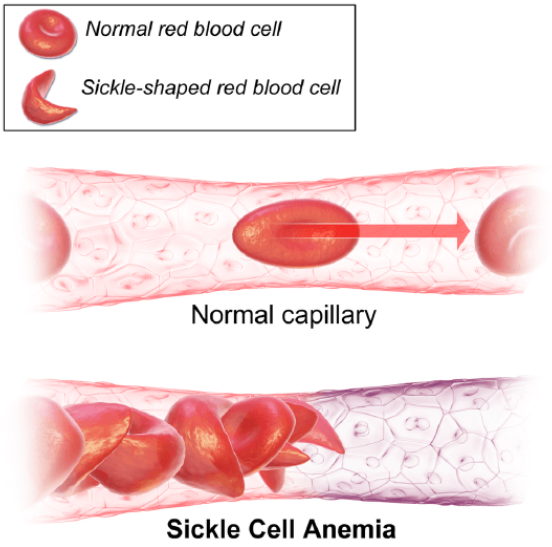


Figure 15.3*I*.1: Sickle-cell anemia. (CC BY-SA 4.0; BruceBlaus).

Causes of sickle-cell anemia

Individuals with sickle-cell disease have inherited from each parent a gene — β^S — encoding the beta chain of hemoglobin. Individuals who inherit only one β^S gene along with the β^A allele have both Hb^A and Hb^S in their red cells. In the malaria-free United States, these heterozygotes are well. In regions where malaria is common, having one of each beta chain gene (β^A and β^S) confers resistance to one of the most dangerous types of malaria (falciparum). This would explain why the Hb^S gene is so prevalent in those regions.

The amino acid sequences of the beta chains of Hb^A and Hb^S have been determined. The beta chains are identical except for the amino acid at position 6 (counting, as always, from the amino terminal). This position is occupied by glutamic acid in Hb^A chains, but in Hb^S beta chains, valine is found there instead.



Figure 15.3*I*.2: Sequence alignment between Hb^A and Hb^S

Why does this single amino acid change in a chain of 146 amino acids so drastically alter the properties of deoxygenated hemoglobin? In switching from glutamic acid to valine, a strongly hydrophilic molecule has been replaced by a strongly hydrophobic one. Position 6 is located at the surface of the beta chain, where it would normally be exposed to water. This switch from a hydrophilic to a hydrophobic region on the surface reduces the solubility of the molecule and promotes the formation of large insoluble aggregates.

The mutation that produces Hb^S is a single-base substitution in which the substitution of a T for an A at the 17th nucleotide of the sense strand of the first exon of the beta chain gene converts a codon for glutamic acid (GAG) to a codon for valine (GTG). Although this change might at first appear trivial, the resulting substitution of valine for glutamic acid so alters the physical properties of hemoglobin that a serious disease is produced in people carrying both genes for the trait.

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15.3J: Serine Proteases

The serine proteases are a family of enzymes that cut certain peptide bonds in other proteins. This activity depends on a set of amino acid residues in the **active site** of the enzyme — one of which is always a serine (thus accounting for their name). In mammals, serine proteases perform many important functions, especially in digestion, blood clotting, and the complement system.

Digestive Enzymes

Three protein-digesting enzymes secreted by the pancreas are serine proteases: chymotrypsin, trypsin and elastase. These three share closely-similar structures (tertiary as well as primary). In fact, their active serine residue is at the same position (**Ser-195**) in all three. Despite their similarities, they have different substrate specificities; that is, they cleave different peptide bonds during protein digestion.

Clotting Factors

Several activated clotting factors are serine proteases, including Factors 10 (X), 11 (XI), and 12 (XII), Thrombin. and Plasmin.

Complement Factors

Several proteins involved in the complement cascade are serine proteases, including

- C1r and C1s
- the **C3 convertases**
  - C4b,2a
  - C3b,Bb

Serpins

Serpins are Serine Protease Inhibitors. Here is a list of a few important serine proteases and the serpins that control them.

Serine Protease	Serpin
Chymotrypsin	alpha-1-antichymotrypsin
Complement factors C1r and C1s	C1 Inhibitor (C1INH)
Elastase (secreted by neutrophils)	alpha-1-antitrypsin
Clotting factor 10 (X)	antithrombin III
Thrombin	antithrombin III
Plasmin	alpha-2-antiplasmin
Trypsin	pancreatic trypsin inhibitor

How Serpins Work

The serpins inhibit the action of their respective serine protease by mimicking the three-dimensional structure of the normal substrate of the protease. The serine protease binds the serpin instead of its normal substrate. This alone would block any further activity by the protease. But the serpin has another trick to play. The protease makes a cut in the serpin leading to the formation of a covalent bond linking the two molecules, a massive allosteric change in the tertiary structure of the serpin, which moves the attached protease to a site where it can be destroyed.

Importance of Serpins

Almost 20% of the proteins found in blood plasma are serpins. Their abundance reflects their importance: putting a stop to proteolytic activity when the need for it is over. This is especially important for the clotting and complement systems where a tiny initial activating event leads to a rapidly amplifying cascade of activity.

Serpin Deficiencies

A number of inherited human diseases are caused by a deficiency of a particular serpin. The deficiency usually results from a mutation in the gene encoding the serpin.

Alpha-1-antitrypsin deficiency

Alpha-1-antitrypsin inactivates the **elastase** secreted by neutrophils. When the lungs become inflamed, neutrophils secrete elastase as a defensive measure. However, it is important to inactivate this elastase as soon as its job is done. That is the function of alpha-1-antitrypsin. Its name, alpha-1-antitrypsin, suggests that it attacks the digestive enzyme, trypsin. In vitro, it does, but in the body, alpha-1-antitrypsin is found in the blood, not the intestine. Inactivation of trypsin in the intestine is the function of another serpin, pancreatic trypsin inhibitor. People with an inherited deficiency of alpha-1-antitrypsin (they are homozygous for a point mutation in its gene) are prone to emphysema. An effective treatment is on the horizon now that genetic engineering has produced goats that secrete human alpha-1-antitrypsin in their milk.

Alpha-1-antitrypsin deficiency can also lead to liver damage. Alpha-1-antitrypsin is synthesized in the liver. However, some mutant versions of the molecule form insoluble aggregates within the liver cells. This mechanism is similar to that of the prion diseases where protein aggregates destroy neurons in the brain. A drug that enhances autophagy protects mice from the liver damage caused by aggregates of mutant alpha-1-antitrypsin.

C1INH deficiency

A deficiency of C1INH produces **hereditary angioedema (HAE)**. In addition to C1r and C1s, C1INH also inhibits several other serine proteases including **kallikrein**, the enzyme responsible for forming the potent vasodilator **bradykinin**. Hence, a deficiency of C1INH can trigger a dangerous swelling (edema) of the airways, as well as of the skin and intestine.

Antiplasmin deficiency

A deficiency in antiplasmin puts the person at risk of uncontrollable bleeding.

Antithrombin deficiency

A deficiency in antithrombin puts the person at risk of spontaneous blood clots, which can lead to a heart attack or stroke. In January 2009, an advisory committee of the U.S. FDA decided that a recombinant human antithrombin (ATryn®) secreted into the milk of transgenic goats was safe for use in therapy.

The Evolution of the Serine Proteases

The close sequence similarity of the various mammalian serine proteases suggests that each is the product of a gene descended by repeated gene duplication from a single ancestral gene.

Other Serine Proteases

Serine proteases and molecules similar to them are found elsewhere in nature.

Subtilisin

Subtilisin is a serine protease secreted by the bacterium *Bacillus subtilis*. Although it has the same mechanism of action as the serine proteases of mammals, its primary structure and tertiary structure are entirely different. An example at the molecular level

of convergent evolution: two molecules acquiring the same function (analogous) but having evolved from different genes.

Acetylcholinesterase

This enzyme is built like and acts like the other serine proteases, but its substrate is the neurotransmitter **acetylcholine**, not a protein. It is found at several types of synapses as well as at the neuromuscular junction — the specialized synapse that triggers the contraction of skeletal muscle. The organophosphate compounds used as insecticides (e.g., parathion) and nerve gases (e.g. Sarin) bind to the **serine** at the active site of acetylcholinesterase blocking its action.

Serpinlike Molecules

Angiotensinogen

This peptide is the precursor of angiotensin II — a major factor in maintaining blood pressure.

Chicken Ovalbumin

This is the major protein in the "white" of the egg (and a favorite antigen in immunological research).

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15.3K: Animal Circulatory Systems

This page outlines the components and challenges of efficient circulatory systems, highlighting the differences across species. It notes that fish have less efficient systems due to pressure loss in gills, while squids use separate pumps. Amphibians possess three-chambered hearts with some blood mixing, whereas lizards have a muscular septum to enhance separation.

An efficient circulatory system has:

- a fluid, e.g., blood, to carry the materials to be transported
- a system of vessels to distribute the blood
- a pump to push the blood through the system
- exchange organs to carry out exchanges between the blood and external environment, e.g.
  - lungs and intestine to add materials to the blood;
  - lungs and kidneys to remove materials from the blood.

- The most crucial demand on the circulatory system is the transport of oxygen and carbon dioxide to and from a gas exchange organ such as lungs or gills and the tissues.
- All exchanges between blood and cells occur in the capillaries.
- The force of the pump that pushes blood through the arteries is dissipated as the blood flows through capillaries. Although capillaries are tiny, the total cross-sectional area of all the capillaries supplied by a single artery is much greater than that of the artery itself. Like a rapid, narrowly-confined stream spreading out over a flat plain, the force and velocity of flow diminish quickly.

This creates a problem:

- If the pump is used to deliver blood with force to the gas exchange organ, little force remains to distribute the oxygenated blood to the tissues.
- If the pump is used to deliver blood with force to the tissues, little force remains to send the deoxygenated blood to the gas exchange organ.

The Fish Heart

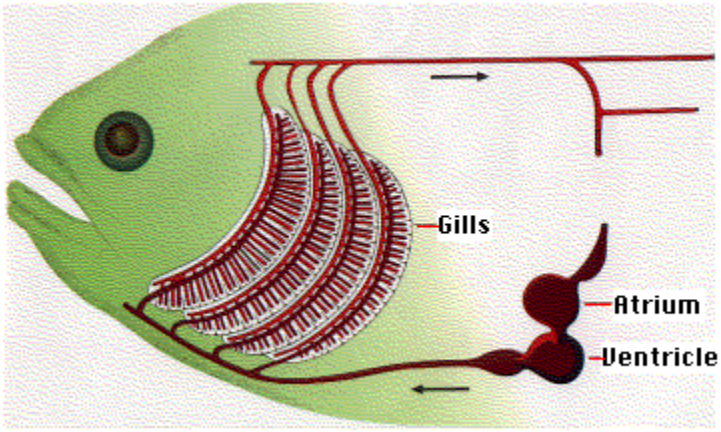


Figure 15.3.11.1 Fish heart

Most fishes have never solved this problem, which is probably why most of them are "cold-blooded".

- Blood collected from throughout the fish's body enters a thin-walled receiving chamber, the **atrium**.
- As the heart relaxes, the blood passes through a valve into the thick-walled, muscular **ventricle**.
- Contraction of the ventricle forces the blood into the capillary networks of the **gills** where gas exchange occurs.
- The blood then passes on to the capillary networks that supply the rest of the body where exchanges with the tissues occur.
- Then the blood returns to the atrium.

While obviously adequate to the fish's needs, this is not a very efficient system. The pressure generated by contraction of the

ventricle is almost entirely dissipated when the blood enters the gills.

The Squid Hearts

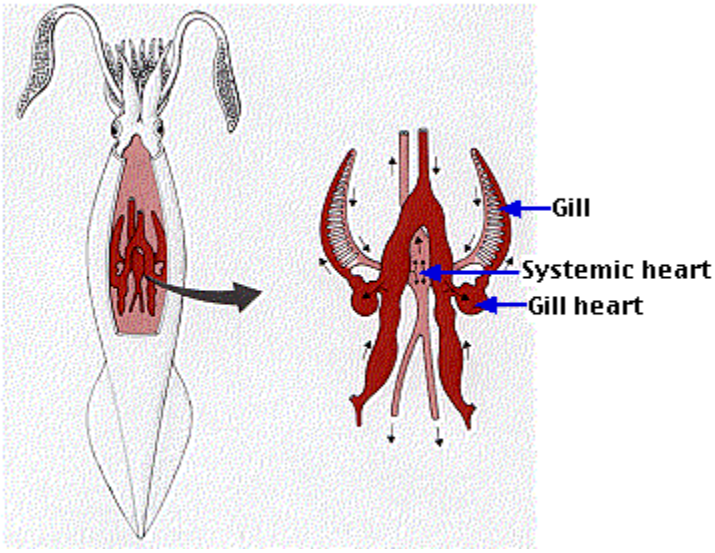


Figure 15.3.11.2 Squid heart

This group of marine invertebrates has solved the problem by having separate pumps:

- two **gill hearts** to force blood under pressure to the gills
- a **systemic heart** to force blood under pressure to the rest of the body

The Frog and Lizard heart

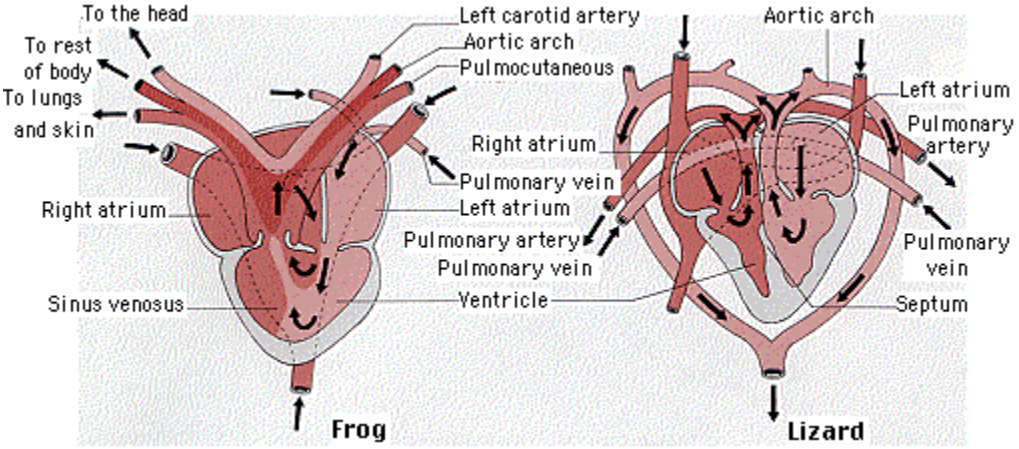


Figure 15.3.11.3 Frog and lizard heart

The Frog Heart

The frog heart has 3 chambers: **two atria** and a single **ventricle**.

- The atrium receives deoxygenated blood from the blood vessels (veins) that drain the various organs of the body.
- The left atrium receives oxygenated blood from the lungs and skin (which also serves as a gas exchange organ in most amphibians).
- Both atria empty into the single ventricle.
- While this might appear to waste the opportunity to keep oxygenated and deoxygenated bloods separate, the ventricle is divided into narrow chambers that reduce the mixing of the two blood.

- So when the ventricle contracts
  - oxygenated blood from the left atrium is sent, relatively pure, into the **carotid arteries** taking blood to the head (and brain)
  - deoxygenated blood from the right atrium is sent, relatively pure, to the **pulmocutaneous arteries** taking blood to the skin and lungs where fresh oxygen can be picked up
  - Only the blood passing into the **aortic arches** has been thoroughly mixed, but even so it contains enough oxygen to supply the needs of the rest of the body
- Note that in contrast to the fish, both the gas exchange organs and the interior tissues of the body get their blood under full pressure.

The Lizard Heart

- Lizards have a muscular **septum** which partially divides the ventricle.
- When the ventricle contracts, the opening in the septum closes and the ventricle is momentarily divided into two separate chambers.
- This prevents mixing of the two bloods.
  - The left half of the ventricle pumps oxygenated blood (received from the left atrium) to the body.
  - The right half pumps deoxygenated blood (received from the right atrium) to the lungs.

Birds, Crocodiles, and Mammals

The septum is complete in the hearts of birds, crocodiles, and mammals providing two separate circulatory systems:

- pulmonary for gas exchange with the environment
- systemic for gas exchange (and all other exchange needs) of the rest of the body

The efficiency that results makes possible the high rate of metabolism on which the endothermy ("warm-bloodedness") of birds and mammals depends.

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15.4: IMMUNE SYSTEM



- Topic hierarchy

15.4.1: 15.4T Allergies

15.4A: Clonal Selection and Immunological Memory

15.4B: Antibody-Antigen Binding

15.4C: B Cells and T Cells

15.4D: Antigen Receptors

15.4E: Histocompatibility Molecules

15.4F: Antigen Receptor Diversity

15.4G: Anatomy of the Immune System

15.4H: T Helper cells

15.4I: Cytotoxic T lymphocytes (CTL)

15.4J: Cell-Mediated Immunity

15.4K: Organ Transplants

15.4L: Bone Marrow Transplants

15.4M: Antigen Presentation

15.4N: The Immunological Synapse

15.4O: Dendritic Cells

15.4P: Passive Immunity

15.4Q: Innate Immunity

15.4R: The Complement System

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15.4U: Asthma

15.4V: AIDS

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15.4.1: 15.4T Allergies

Immunologists, as well as the general public, use the term allergy in several different ways. An allergy is a harmful immune response elicited by an antigen that is not itself intrinsically harmful. Examples:

- The windblown pollen released by orchard grass has no effect on me but produces a violent attack of hay fever (known to physicians as **allergic rhinitis**) in my wife.
- She, on the other hand, can safely handle the leaves of **poison ivy** while if I do so, I break out in a massive skin rash a day or two later.

Antigens that trigger allergies are often called **allergens**.

Four different immune mechanisms can result in allergic responses.

- Immediate Hypersensitivities: These occur quickly after exposure to the allergen. They are usually mediated by antibodies of the IgE class. Examples include hay fever, hives and asthma.
- Antibody-Mediated Cytotoxicity: Cell damage caused by antibodies directed against cell surface antigens. Hence a form of autoimmunity. Examples include Hemolytic disease of the newborn (Rh disease) and Myasthenia gravis (MG)
- Immune Complex Disorders: Damage caused by the deposit in the tissues of complexes of antigen and their antibodies. Examples include Serum sickness and Systemic lupus erythematosus (SLE)
- Cell-Mediated Hypersensitivities: These reactions are mediated by $CD4^+$ T cells. Examples:

The rash produced following exposure to poison ivy. Because it takes a day or two for the T cells to mobilize following exposure to the antigen, these responses are called **delayed-type hypersensitivities** (**DTH**). Those, like poison ivy, that are caused by skin contact with the antigen are also known as contact sensitivities or **contact dermatitis**.

- certain **autoimmune diseases**, including
  - Type 1 diabetes mellitus
  - Multiple sclerosis (MS)
  - Rheumatoid arthritis (RA)

Immediate Hypersensitivities

Local Anaphylaxis

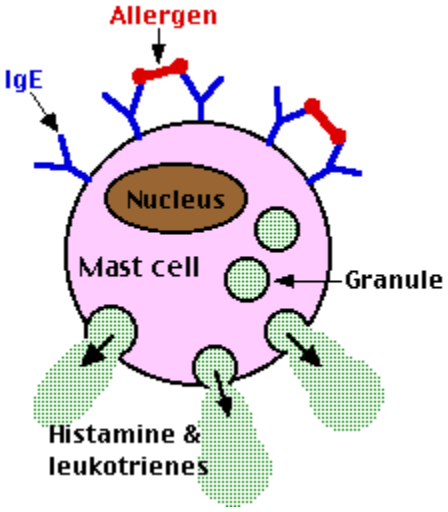


Figure 15.4.20.1 Mast cell

The constant region of IgE antibodies (shown in blue) has a binding site for a receptor present on the surface of basophils and their tissue-equivalent the **mast cell**. These cell-bound antibodies have no effect until and unless they encounter allergens (shown in red) with epitopes that can bind to their antigen-binding sites. When this occurs, the mast cells to which they are attached

- explosively discharge their granules by exocytosis. The granules contain a variety of active agents including **histamine**;
- synthesize and secrete other mediators including **leukotrienes** and **prostaglandins**.

Release of these substances into the surrounding tissue causes **local anaphylaxis**: swelling, redness, and itching. In effect, each IgE-sensitized mast cell is a tiny bomb that can be exploded by a particular antigen. The most common types of local anaphylaxis are:

- **allergic rhinitis** (**hay fever**) in which airborne allergens react with IgE-sensitized mast cells in the nasal mucosa and the tissues around the eyes;
- bronchial **asthma** in which the allergen reaches the lungs either by inhalation or in the blood
- **hives** (physicians call it **urticaria**) where the allergen usually enters the body in food.

Leukotrienes are far more potent than histamine in mediating these reactions.

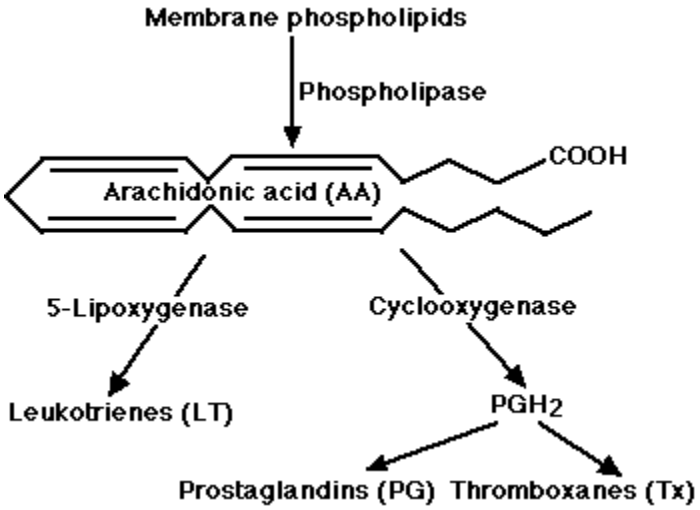


Figure 15.4.20.2 Arachidonic acid

Leukotrienes and prostaglandins are derivatives of **arachidonic acid** (**AA**) an unsaturated fatty acid produced from membrane phospholipids. The principal pathways of arachidonic acid metabolism are

- the **5-lipoxygenase** pathway, which produces a collection of **leukotrienes** (LT) and
- the **cyclooxygenase** pathway, which yields a number of **prostaglandins** (PG) and **thromboxanes** (Tx).

All three are synthesized by monocytes and macrophages. Mast cells and basophils generate a mixture of leukotrienes. The products of both pathways act in concert to cause inflammation with prostaglandins producing fever and pain. Aspirin, ibuprofen, and certain other nonsteroidal anti-inflammatory drugs (NSAIDs) achieve their effects (fever and pain reduction) by blocking the activity of cyclooxygenase.

Some people respond to environmental antigens (e.g., pollen grains, mold spores) with an unusually vigorous production of **IgE antibodies**. Why this is so is unclear; heredity certainly plays a role. In any case, the immune system of these people is tilted toward the production of T helper cells of the **Th2** subtype. These release **interleukin 4** (IL-4) and **interleukin 13** (IL-13) on the B cells that they "help". These lymphokines promote class switching in the B cell causing it to synthesize IgE antibodies.

An inherited predisposition to making IgE antibodies is called **atopy**. Atopic people are apt to have higher levels of circulating IgE (up to 12 µg/ml) than is found usually (about 0.3 µg/ml). Whereas only 20–50% of the receptors on mast cells are normally occupied by IgE, all the receptors may be occupied in atopic individuals.

Skin Testing

When the problem allergen is not obvious, it can often be identified by skin testing. A panel of suspected allergens is injected into separate sites in the skin and each site is observed for the development of a "**wheal and flare**" reaction. The wheal is a sharply delineated soft swelling surrounded by the flare - a reddened area. Both are caused by the release of leukotrienes at the site, which increase the flow of blood to the site making it swollen and red.

A positive skin test occurs within minutes or even seconds (in contrast to patch testing for DTH responses).

Systemic Anaphylaxis

Some allergens can precipitate such a massive IgE-mediated response that a life-threatening collapse of the circulatory and respiratory systems may occur.

Frequent causes:

- insect (e.g., bee) stings
- many drugs (e.g., penicillin)
- a wide variety of foods. Egg white, cow's milk, and nuts are common offenders in children; in fact, some school systems in the US now ban peanuts and peanut-butter sandwiches when they have a student at risk of systemic anaphylaxis from exposure to peanuts. Fish and shellfish are frequent causes of anaphylaxis in adults.

Treatment of systemic anaphylaxis centers on the quick administration of adrenaline, antihistamines, and if shock has occurred then intravenous fluid replacement.

An example of systemic anaphylaxis

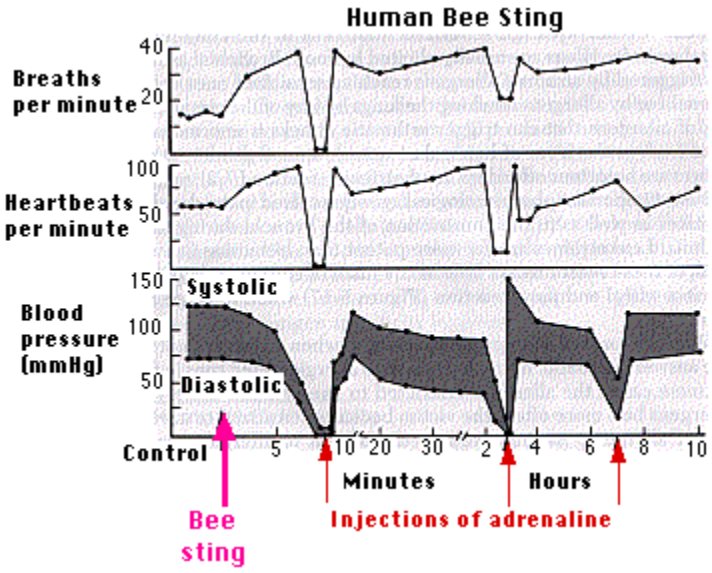


Figure 15.4.20.3 Reaction to bee sting

The three graphs show the physiological responses of a physician (Dr. Vick) stung by a single bee while on a picnic with coworkers (fortunately some with medical training!). Dr. Vick required cardiac massage and intravenous injections of adrenaline at the times shown. He and his colleagues worked in a laboratory studying bee venom, but prior to this episode he had no idea that he had developed such extreme susceptibility. [Courtesy of Dr. J. Vick from L. M. Lichtenstein, "Allergic Responses to Airborne Allergens and Insect Venoms", *Fed. Proc.* **36:**1727, 1977.]

Desensitization

So far, the most effective preventive for IgE-mediated allergies is to inject the patient with gradually-increasing doses of the allergen itself. The goal is to shift the response of the immune system away from Th2 cells in favor of Th1 cells. Unfortunately,

this therapy takes a long time and the results are too often disappointing. Clinical trials are now underway to test the safety and efficacy of a complex of ragweed pollen allergen with chemically-modified DNA. This complex binds to the immune receptor TLR-9 causing a shift of the immune response from Th2 to Th1 much more rapidly than desensitization by the allergen alone.

Anti-IgE Antibodies

IgE molecules bind to mast cells and basophils through their constant region. If you could block this region, you could interfere with binding — hence sensitization of — these cells. Humanized monoclonal antibodies specific for the constant region of IgE are in clinical trials. They have shown some promise against asthma and peanut allergy, but such treatment will probably have to be continued indefinitely (and will be very expensive).

IgE-Independent Allergic Reactions

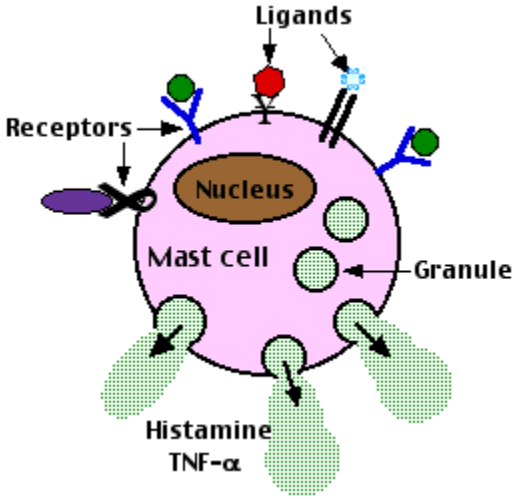


Figure 15.4.20.4 Mast cell

Mast cells have surface receptors in addition to IgE molecules. Binding of ligands to these other receptors can also trigger degranulation and immediate anaphylactic responses. Some culprits are:

- pathogen-associated molecular patterns (PAMPs)
- substance P
- some components of wasp venoms
- some antibiotics

Antibody-Mediated Cytotoxicity

In these disorders, the person produces antibodies directed against antigens present on the surface of his or her own cells. Thus these qualify as autoimmune disorders. Some examples:

- **hemolytic disease of the newborn** (**Rh disease**)
- **immune hemolytic anemia**
- **immune thrombocytopenic purpura**
- **myasthenia gravis** (**MG**)
- **thyrotoxicosis** (**Graves' disease**)
- pemphigus and pemphigoid, in which the antibodies are directed against the proteins in desmosomes (pemphigus) or hemidesmosomes (pemphigoid).
- Goodpasture's Syndrome

Binding of antibodies to the surface of the cell can result in:

- phagocytosis of the cell
- lysis of the cell

- damage to molecules on the cell surface (e.g., myasthenia gravis)
- activation of cell-surface receptors (e.g., thyrotoxicosis)

Hemolytic Disease of the Newborn (Rh Disease)

Rh antigens are expressed at the surface of red blood cells. During pregnancy, there is often a tiny leakage of the baby's red blood cells into the mother's circulation. If the baby is Rh-positive (having inherited the trait from its father) and the mother Rh-negative, these red cells will cause her to develop antibodies against the Rh antigen. The antibodies, usually of the IgG class, may not develop fast enough to cause problems for that child, but can cross the placenta and attack the red cells of a subsequent Rh^+ fetus. This destroys the red cells producing anemia and jaundice. The disease may be so severe as to kill the fetus or even the newborn infant.

Although certain other red cell antigens (in addition to Rh) sometimes cause problems for a fetus, an **ABO** incompatibility does not. Why is an Rh incompatibility so dangerous when ABO incompatibility is not? It turns out that most anti-A or anti-B antibodies are of the IgM class and these do **not** cross the placenta. In fact, an **Rh^-/type O** mother carrying an **Rh^+/type A, B, or AB** fetus is resistant to sensitization to the Rh antigen. Presumably her anti-A and anti-B antibodies destroy any fetal cells that enter her blood before they can elicit anti-Rh antibodies in her.

This phenomenon has led to an extremely effective preventive measure to avoid Rh sensitization. Shortly after each birth of an Rh^+ baby, the mother is given an injection of anti-Rh antibodies. The preparation is called **Rh immune globulin** (**RhIG**) or **Rhogam**. These passively acquired antibodies destroy any fetal cells that got into her circulation before they can elicit an active immune response in her.

Rh immune globulin came into common use in the United States in 1968, and within a decade the incidence of Rh hemolytic disease became very low.

Immune Hemolytic Anemia

Some people synthesize antibodies against their own red blood cells, and these may lyze the cells producing anemia. Infections, cancer, or an autoimmune disease like **systemic lupus erythematosus** (**SLE**) are often involved. Many drugs (e.g. penicillin, quinidine) can also trigger the disorder. In these cases, stopping the drug usually brings about a quick cure.

Immune Thrombocytopenic Purpura

This is an autoimmune disorder in which the patient develops antibodies against his or her own platelets (thrombocytes). The life span of the platelets may be reduced from the normal of 8 days to as little as 1 hour, and platelet counts may drop from a normal of 140,000–440,000/µl to 20,000/µl or less. This greatly interferes with normal clotting, causing

- external bleeding (e.g., from the nose)
- internal bleeding into the skin causing purple patches (called purpura)

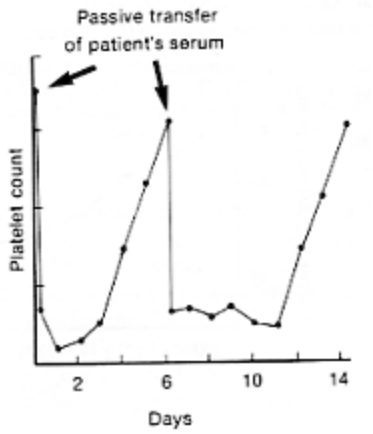


Figure 15.4.20.4 Platelet count graph

The fact that antibodies are the culprit was dramatically demonstrated by using a patient's serum to passively — but only temporarily —transfer the disorder to a normal recipient. The graph shows the decline and recovery in the platelet count of a normal human subject receiving two transfusions of serum from a patient with thrombocytopenic purpura. [From W. J. Harrington et al., *J. Lab. Clin. Med.* **38**:1, 1951.]

Often no cause of the disorder can be found (the physicians call it "idiopathic"). Some cases are triggered by drugs like quinine, aspirin, digitoxin, and sulfa drugs. These cases can be cured by stopping the drug. The idiopathic cases can sometimes be helped by giving corticosteroids and/or removing the patient's spleen. Rituximab, a monoclonal antibody directed against B cells is also used. If these treatments are inadequate, attempts can be made to increase the platelet count by giving synthetic agonists (e.g., Romiplastin [Nplate®]) that stimulate the production of thrombopoietin.

Myasthenia Gravis (MG)

The hallmark of this autoimmune disorder is weakness of the skeletal muscles, especially those in the upper part of the body. It is caused by antibodies that attack the **acetylcholine (ACh) receptors** at the subsynaptic membrane of **neuromuscular junctions**. As the number of receptors declines, the ACh released with the arrival of a volley of nerve impulses is inadequate to generate end-plate potentials (EPPs) of the normal size. After repeated stimulation, the EPPs fail to reach the threshold needed to generate an action potential and the muscle stops responding.

The signs and symptoms of myasthenia gravis can be quickly but only temporarily relieved by injecting a drug that inhibits the action of cholinesterase. This prolongs the action of ACh at the neuromuscular junction. The immunosuppressant action of corticosteroids, like prednisone, can provide long-term improvement for patients. The exclusive role of antibodies (of the IgG class) in this disorder is demonstrated by the presence of the disease in the newborn babies of mothers with the disorder. As these antibodies, which the fetus had received from the mother's circulation, disappear (in 1–2 weeks), so do all signs of the disease.

Thyrotoxicosis (Graves' disease))

In this disorder, the patient has antibodies that bind to the **TSH receptors** on the thyroxine-secreting cells of the thyroid. These antibodies mimic the action of TSH itself (thus they behave as a TSH agonist) and trigger secretion of thyroxine (T_4) and T_3 by the thyroid gland. The role of antibodies (of the IgG class) in this disorder is demonstrated by the presence of the disease in the newborn babies of mothers with the disorder. As these antibodies, which the fetus had received from the mother's circulation, disappear (in 1–2 weeks), so do all signs of the disease.

Immune Complex Disorders

While binding of antibody to antigen is often a helpful — even life-saving — response, in some circumstances it causes pathological changes.

Serum Sickness

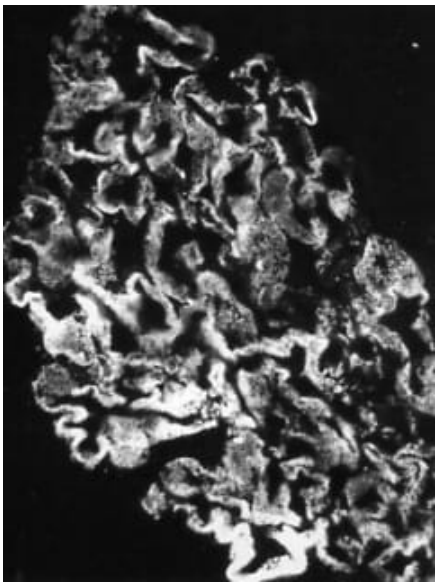


Figure 15.4.20.5 Irregular, lumpy deposits of immune complexes in a glomerulus courtesy of Dr. Frank J. Dixon

In passive immunization, an antiserum containing needed antibodies is injected into the patient. At one time, these antisera were prepared by immunizing horses or sheep. While they did their intended work (usually to provide immediate protection to a person exposed to diphtheria or tetanus), they also often later lead to a syndrome called serum sickness. The patient developed fever, hives, arthritis and protein in the urine. After a week or two, the symptoms would disappear spontaneously.

Serum sickness is caused by the many extraneous proteins present in the antiserum. Being foreign to the recipient, an **active immunity** develops against these proteins. The resulting antibodies bind to them forming **immune complexes**. These are carried by the blood and deposited in the walls of blood vessels as well as in the glomeruli of the kidneys (see figure).

Antigen-antibody complexes

- bind to Hageman factor — one (XII) of the blood clotting factors. This activates inflammatory kinins.
- bind to a system of serum proteins collectively known as **complement**. This generates
  - complement factor **C3a**, an anaphylatoxin which activates basophils and mast cells and causes them to release their histamine and leukotrienes producing inflammation.
  - complement factor **C5a**, another anaphylatoxin which also attracts neutrophils to the site.

Thanks to nearly universal active immunization against both tetanus and diphtheria, serum sickness is now quite rare. However, kidney damage (called **glomerulonephritis**) produced by deposits of immune complexes is found in some persistent infections.

Examples:

- the protozoans that cause malaria
- the flatworms that cause schistosomiasis and the filarial worms that cause elephantiasis and other diseases in humans.
- the virus that causes hepatitis B.

In these cases, the continued presence of the pathogen provides a renewable source of antigen to combine with antibodies synthesized by the host resulting in deposits of immune complexes.

Systemic Lupus Erythematosus (SLE)

Humans with SLE develop (for unknown reasons) antibodies against a wide variety of self components:

- their own double-stranded DNA
- nucleosomes
- red blood cells

- platelets
- NMDA receptors in the brain
- even their own IgG molecules. (These "anti-antibodies" are called **rheumatoid factors**. They are also found in people with **rheumatoid arthritis** (hence the name) and, for a time, in people with **mononucleosis**.)

In all these cases of autoimmunity, immune complexes form and are deposited in the skin, joints, and kidneys where they initiate inflammation.

Farmer's Lung

Repeated exposure to airborne organic particles, like mold spores, can elicit formation of antibodies. When these interact with inhaled antigen, inflammation of the alveoli occurs. The sufferer develops a cough, fever, and difficulty in breathing. Once removed from the source of antigen, the attack subsides within a few days.

Farmers exposed to moldy hay often develop this problem (technically known as **extrinsic allergic alveolitis**). Sugarcane workers, cheese makers, mushroom growers, pigeon fanciers, and a number of other occupational or hobby groups are apt to develop allergic alveolitis from exposure to the spores and dusts associated with their activities.

Cell-Mediated Hypersensitivities

Cell-mediated hypersensitivities can occur with extrinsic antigens or with internal ("self") antigens.

Extrinsic Antigens

The most common example of cell-mediated hypersensitivity to external antigens is the **contact dermatitis** caused in some people when their skin is exposed to a chemical to which they are allergic. Some examples:

- the catechols found in poison ivy, poison oak, and poison sumac
- nickel (often used in jewelry)
- some dyes
- certain organic chemicals used in industry

In every case, these simple chemicals probably form covalent bonds with proteins in the skin, forming the antigen that initiates the immune response. Dendritic cells in the skin take up the complex, process it, and "present" it to $CD4^+$ T cells in nearby lymph nodes. Because it takes a day or two for the $CD4^+$ T cells to mobilize to the affected area of skin, these cases are examples of delayed-type hypersensitivity (DTH).

When a patient is unsure of what chemical is causing the dermatitis, the physician can try a **patch test**. Pieces of gauze impregnated with suspected allergens are placed on the skin. After 48 hours, they are removed and each site is examined for a positive response (a reddened, itching, swollen area).

Intrinsic ("self") Antigens

Cell-mediated hypersensitivities to "self" cause autoimmune diseases. Examples:

- Type 1 diabetes mellitus
- Multiple sclerosis (MS)
- Rheumatoid arthritis (RA)

Type 1 diabetes mellitus

In this disease, T cells initiate the destruction of the insulin-producing beta cells of the islets of Langerhans in the pancreas. The chief culprits are $CD8^+$ cytotoxic T lymphocytes (CTL) aided and abetted by $CD4^+$ helper T cells of the Th1 subset. Although antibodies against beta cell antigens are also found, these appear to be a secondary effect. Evidence: a diabetic boy with X-linked

agammaglobulinemia so unable to make any antibodies at all.

Multiple Sclerosis (MS)

In this case, T cells — again mostly Th1 cells — initiate an attack that destroys the myelin sheath of neurons. As the disease progresses, other cells (e.g. macrophages) as well as antibodies participate in causing the damage.

Rheumatoid Arthritis (RA)

In this disorder, antibodies and T cells — again probably Th1 cells — react to antigens in the joints and release **tumor necrosis factor-alpha** (**TNF-α**) with resulting inflammation and damage to the joints.

A genetically engineered fusion protein consisting of the **TNF receptor** fused to the constant region of human IgG1 has shown promise as a treatment for RA. Given by injection, the fusion protein binds TNF-α and interferes with its action.

Autoimmune disorders are more common in females than in males

Graves' disease, systemic lupus erythematosus (SLE), multiple sclerosis, and rheumatoid arthritis are all more common in women than in men. The sex bias ranges from 9:1 for SLE to >2:1 for multiple sclerosis and rheumatoid arthritis. **Why?**

The answer is unclear, but hormones are probably involved.

A few clues:

- In mice susceptible to Type 1 diabetes, testosterone seems to play a key role.
  - Castration causes male mice to become as susceptible as females.
  - Giving androgens like testosterone to females protects them.
- High levels of estrogen and progesterone **suppress** Th1 responses (cell-mediated immunity).
- Pregnant women — with extra-high levels of these hormones — produce large numbers of immunosuppressive regulatory T cells. Together these two responses may account for the improvement that often occurs in multiple sclerosis and rheumatoid arthritis during pregnancy (an improvement that ends after birth).
- High levels of these hormones **promote** Th2 responses (antibody-mediated immunity). SLE results from antigen-antibody complexes and so it is not surprising that pregnancy does not help — and in some women actually exacerbates — this autoimmune disorder.

The Hygiene Hypothesis

Allergies, like asthma and hay fever, and some autoimmune diseases are more common in regions with good sanitation.

For example,

- Crohn's disease, an inflammation of the small intestine and
- ulcerative colitis, an inflammation of the large intestine

are rare in developing countries with poor sanitation but have become more common in developed regions with good sanitation.

Why?

No one knows for certain, but one intriguing possibility, called the **hygiene hypothesis**, is that infection by parasitic worms (helminths) shifts the balance of the immune response:

- promoting regulatory T cells (Treg) with their anti-inflammatory cytokines IL-10 and TGF-β and
- inhibiting pro-inflammatory effector T cells, e.g., Th17 cells.

Small clinical trials of feeding whipworm (a nematode) eggs to patients with Crohn's disease or ulcerative colitis have begun. After the eggs develop into mature worms in their intestine, most patients showed marked improvement of their symptoms.

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15.4A: Clonal Selection and Immunological Memory

This page explains how the immune system responds to antigens through B and T lymphocytes, which have specific receptors. Clonal selection leads to the activation and proliferation of B cells into plasma and memory cells in lymph nodes and spleen. It details the role of dendritic cells in presenting antigens and discusses graft rejection, highlighting the differences between primary and secondary immune responses, with memory cells facilitating faster reactions in subsequent exposures.

The ability of the immune system to respond to an antigen exists **before** it ever encounters that antigen. The immune system relies on the prior formation of an incredibly diverse population of:

- **B cells** (B lymphocytes) each with its surface covered with thousands of identical copies of a **receptor for antigen** (the B-cell receptor for antigen = **BCR**)
- **T cells** (T lymphocytes) each with its surface covered with thousands of identical copies of a T-cell receptor for antigen (**TCR**)

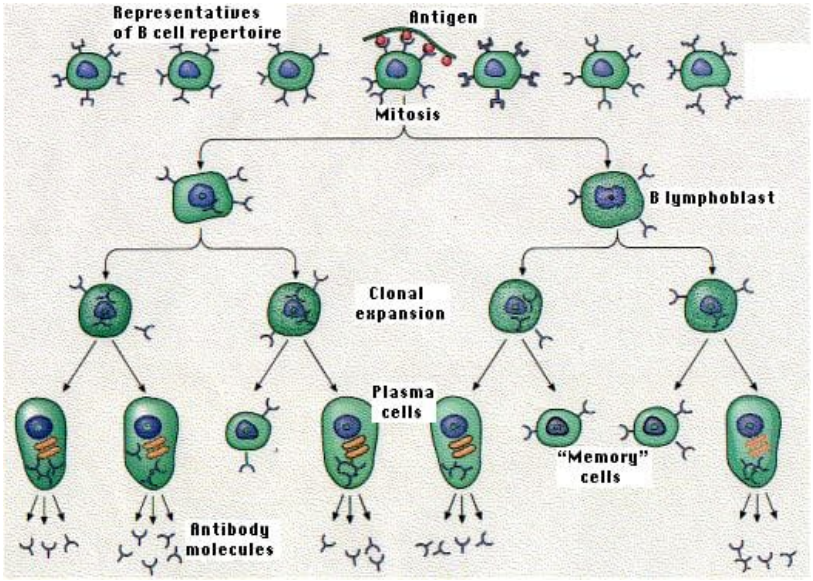


Figure 15.4.1.1 Clonal selection

The above figure illustrates the activation of the one B cell in a pool of B cells whose BCR is specific for an epitope (small dark spheres) on the antigen. This phenomenon is called **clonal selection** because it is antigen that selects particular lymphocytes for clonal expansion. Clonal selection leads to the eventual production of:

- A pool of antibody-secreting plasma cells. **Plasma cells** are B-cells that have tooled up (e.g., forming a large endoplasmic reticulum) for massive synthesis and secretion of an antibody. The antibody is the secreted version of the BCR. (For clarity, each BCR is shown with a single binding site for the epitope; actually, the BCRs are IgM and each has 10 identical binding sites.
- A pool of **"memory" cells**. These are B lymphocytes with receptors of the same specificity as those on the original activated B cell.

How B cells and T cells meet antigens

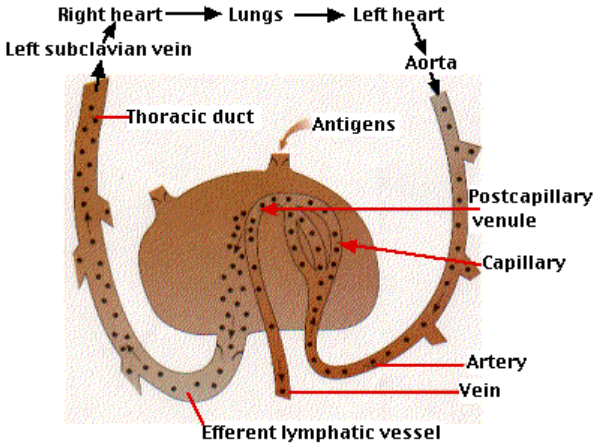


Fig.15.4.1.2 Postcapillary venule

What is the probability that those few lymphocytes able to bind to a particular epitope will actually encounter the antigen carrying that epitope?

Surprisingly, it is quite high because both B cells and T cells migrate in and out of **lymph nodes** and the spleen. Lymph nodes serve as lymph filters, trapping foreign matter that gains access to the tissues. It has been shown that as much as 99% of the bacteria entering a node are removed by it. Similarly, the spleen traps antigens that gain access to the blood. Even if an invader fails to enter either lymphatic or blood vessels, its antigens can still reach lymph nodes and spleen carried there by dendritic cells that

- engulf the antigen in the tissues
- migrate in the lymphatic vessels to nearby lymph nodes or spleen
- process the antigen and "present" it to T cells and also B cells

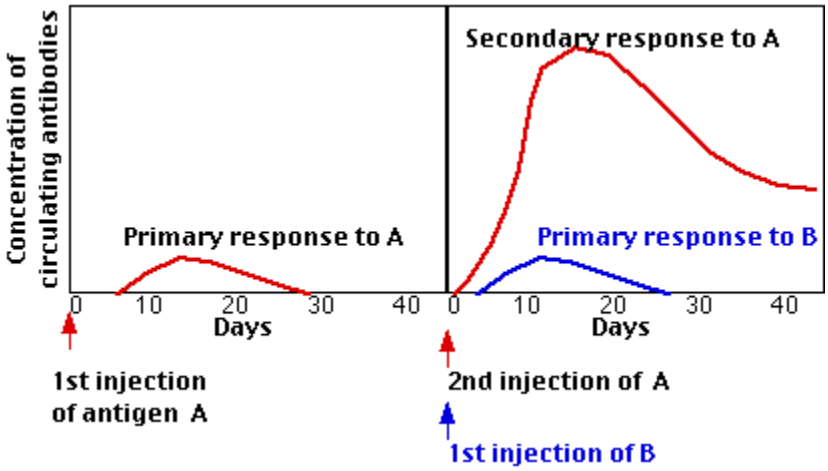


Figure 15.4.1.4 Graft rejection in a mouse

Graft rejection is a form of cell-mediated immunity. If a piece of skin from a mouse of one strain (**B**) is grafted onto the flank of a mouse of a second strain (**A**), the graft does well at first. Blood vessels from the host grow into it, and it functions normally. After some 10–14 days, however, the blood supply to the graft breaks down and the graft degenerates. Finally it is sloughed off like an old scab. This is called a **first-set rejection**. That the graft rejection is an immune response is demonstrated by now grafting the mouse with two pieces of skin a repeat of skin from strain **B** and skin from another strain (**C**)

The results are quite different.

- The B skin may not even survive long enough to acquire a blood supply. It is rejected in a much shorter period (less than a week). This **"second-set"** phenomenon is the T-cell equivalent of the secondary "memory" response of B cells. Its **specificity** and **memory** is shown by the fact that
- The graft of **C skin** is rejected in the normal "first-set" period.

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15.4B: Antibody-Antigen Binding

Antibodies are proteins synthesized and secreted by B cells that bind to antigens. Most antigens are macromolecules: proteins, polysaccharides, even DNA and RNA. The interaction occurs by noncovalent forces (like that between enzymes and their substrate) between the antigen-combining site on the antibody and a portion of the antigen called the **antigenic determinant** or **epitope**.

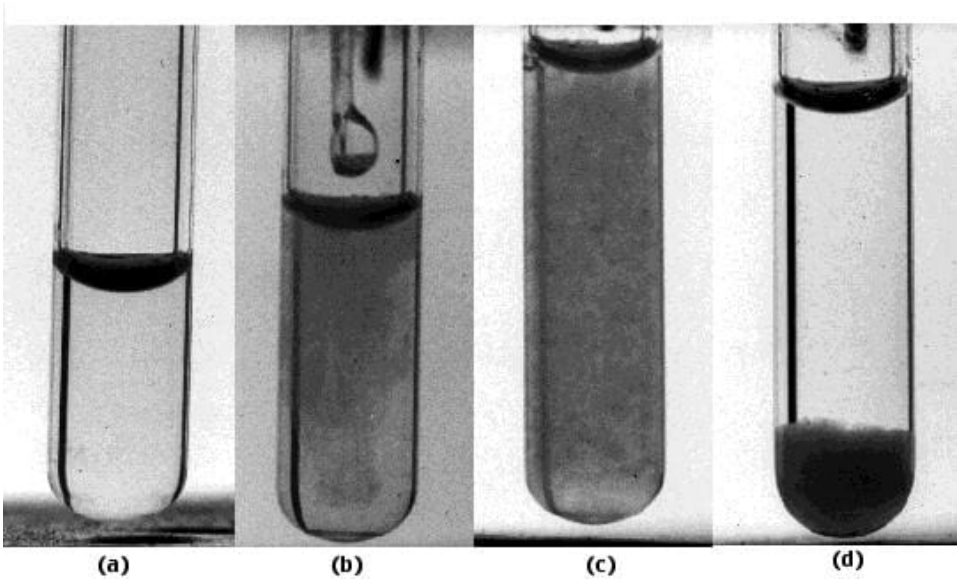


Figure 15.4.2.1 Precipitation between antibodies and antigen

These photos show one type of interaction — precipitation — between antibodies and antigen.

a. The tube contains antibodies to the Type III pneumococcal polysaccharide isolated from the capsule surrounding the bacteria.
b. A solution of the polysaccharide is added.
c. The formation of insoluble antigen-antibody complexes is revealed by the almost instantaneous appearance of turbidity.
d. After an hour, the complexes settle out as a precipitate. If the proportion of antigen to antibody in the mixture is selected properly, the fluid above the precipitate will be devoid of both.

In the human body, this binding can literally be life-saving. The capsule that surrounds pneumococci protects them from phagocytosis. Pneumococci that fail to make a capsule — "R" forms — do not cause disease. If the appropriate antibodies are present in the body, they combine with the capsule. Coated with protein instead of polysaccharide, the pneumococci are now easy to ingest.

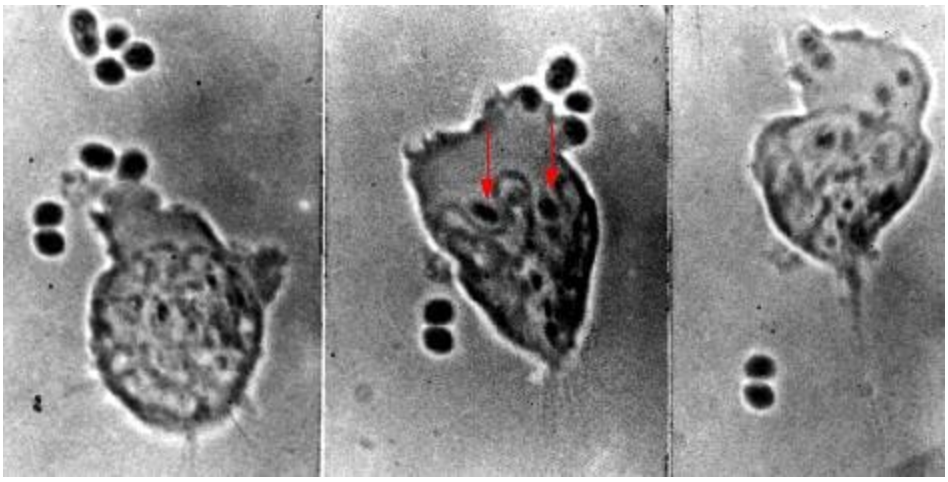


Figure 15.4.2.2 Phagocytosis of antibody-coated pneumococci courtesy W. B. Wood, M. R. Smith, and B. Watson, Journal of Experimental Medicine **84**:387, 1946

These photomicrographs show phagocytosis of antibody-coated pneumococci.

- Left: A neutrophil extends a pseudopod toward two pneumococci.
- Center: these bacteria have been engulfed (arrows), and the neutrophil is beginning to engulf four more pneumococci at the upper right.
- Right: Two pneumococci have escaped.

In the days before antibiotics, the start of antibody production by the immune system of the patient marked the turning point in the progression of the disease.

The Antigen-Combining Site

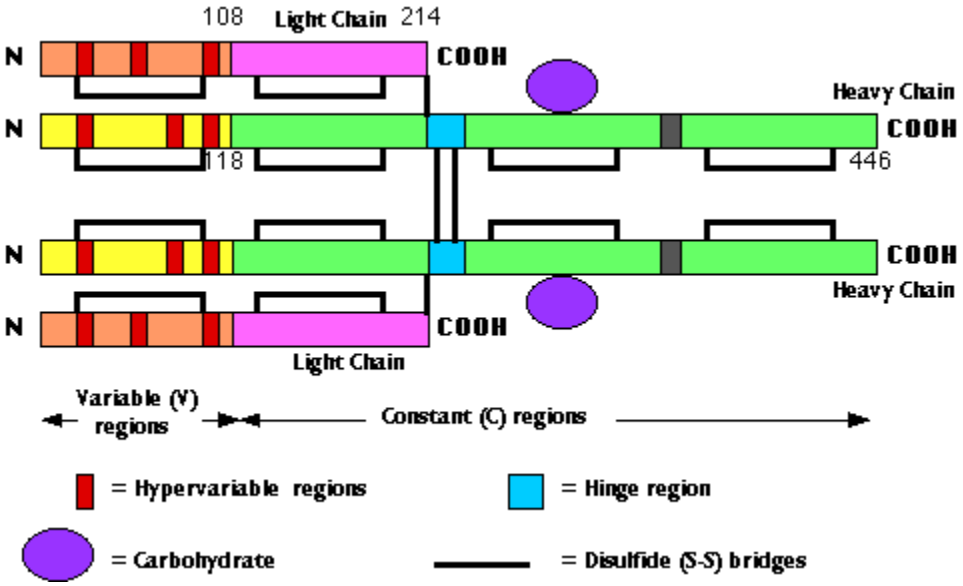


Figure 15.4.2.3 IgG antibody

The fig. 15.4.2.3 shows the primary structure of an IgG antibody. Different IgG antibodies differ most markedly at the so-called hypervariable regions (shown in red):

- three in the heavy chain
- three in the light chain

In the three-dimensional (tertiary) structure of the molecule, the 6 hypervariable regions are brought close together and make up

the antigen-combining site. For this reason, the hypervariable regions are also called **complementarity determining regions** (**CDR**s).

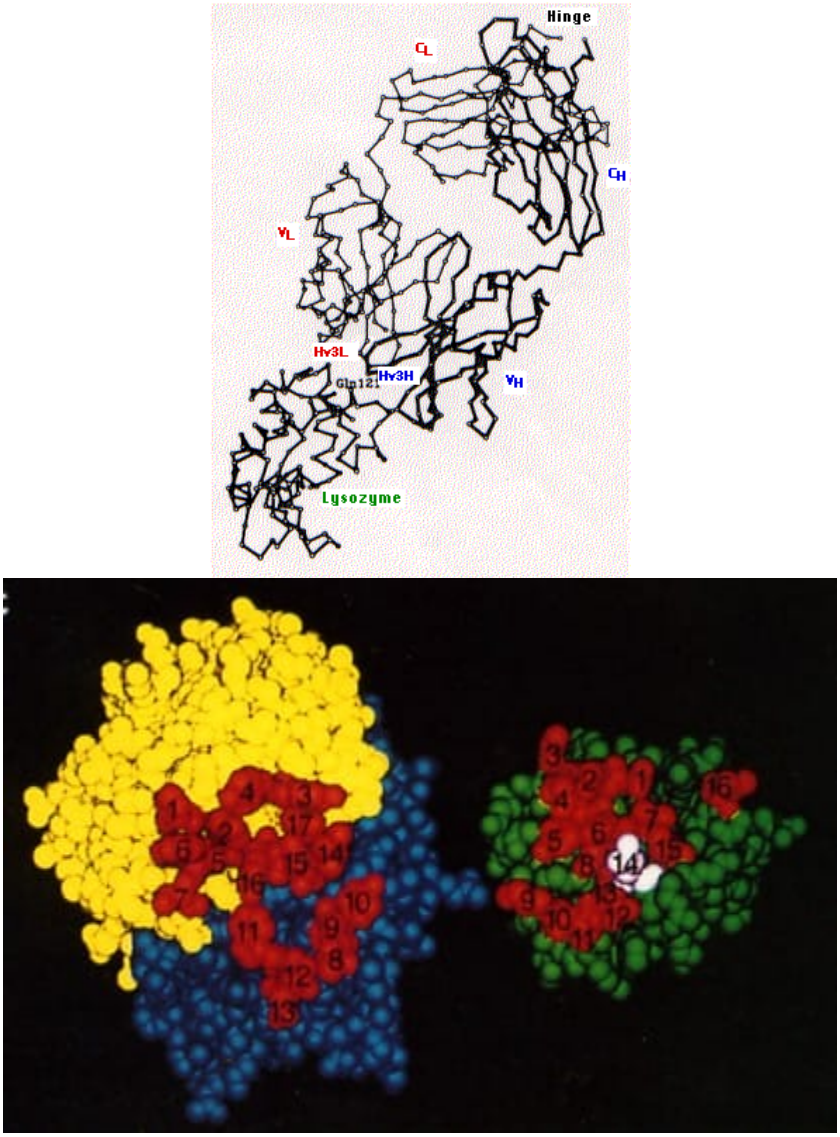


Figure 15.4.2.5 Space-filling model of the same antibody courtesy of A. G. Amit

The fig. 15.4.2.4 shows the tertiary structure of one arm of an antibody specific for the lysozyme found in hen's egg white (upper right) bound to lysozyme (lower left).

The following are indicated on the diagram:

- constant region of the light chain (C_L) and the first constant region of the heavy chain (C_H)
- variable regions of the heavy (V_H) and light chains (V_L)
- the third hypervariable region of the heavy (Hv3H) and light (Hv3L) chains. The importance of the pair of third hypervariable or complementarity determining regions in binding the epitope is typical of both antibodies and of T cell receptors for antigen. This makes good sense as the opportunities for genetic diversity at those sites is far greater than for the first and second CDRs.
- Gln121 is a glutamine that is a dominant feature of the epitope on lysozyme

The fig. 15.4.2.5 is a space-filling model of the same antibody with the light chain in yellow, the heavy chain in blue. The view is looking down on the epitope-binding surface (left) and the epitope on lysozyme (right). In the antigen-antibody complex, the two surfaces fit snugly together. The 17 amino acid residues that contact lysozyme in the antigen-antibody complex are numbered

(1–7 on the L chain, 8–17 on the H chain. The 16 amino acid residues of lysozyme that contact the antibody; that is, that make up its epitope are also numbered. Number 14 is Gln-121. The complementarity of the antigen-binding site and the epitope, their respective shapes and the opportunities for multiple noncovalent interactions determine how strongly the two bind together. The **strength** of the binding of an antibody to its antigen is called its **affinity.**

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15.4C: B Cells and T Cells

Lymphocytes are one of the five kinds of white blood cells (leukocytes) that circulate in the blood. Although mature lymphocytes all look pretty much alike, they are extraordinarily diverse in their functions. The most abundant lymphocytes are B lymphocytes (B cells) and T lymphocytes (T cells). B cells are produced in the **bone** marrow. The precursors of T cells are also produced in the bone marrow but leave the bone marrow and mature in the **thymus** (which accounts for their designation). Each B cell and T cell is specific for a particular antigen. What this means is that each is able to bind to a particular molecular structure. The specificity of binding resides in a receptor for antigen: the B cell receptor (BCR) for antigen and the T cell receptor (TCR) respectively.

Both BCRs and TCRs share these properties:

- They are integral membrane proteins.
- They are present in thousands of identical copies exposed at the cell surface.
- They are made before the cell ever encounters an antigen.
- They are encoded by genes assembled by the recombination of segments of DNA.
- They have a unique binding site.
- This site binds to a portion of the antigen called an antigenic determinant or epitope.
- The binding, like that between an enzyme and its substrate depends on complementarity of the surface of the receptor and the surface of the epitope.
- The binding occurs by non-covalent forces (again, like an enzyme binding to its substrate).
- Successful binding of the antigen receptor to the epitope, if accompanied by additional signals, results in:
  - stimulation of the cell to leave G_0 and enter the cell cycle.
  - Repeated mitosis leads to the development of a clone of cells bearing the same antigen receptor; that is, a clone of cells of the identical specificity.

BCRs and TCRs differ in their structure, the genes that encode them and the type of epitope to which they bind.

B Cells

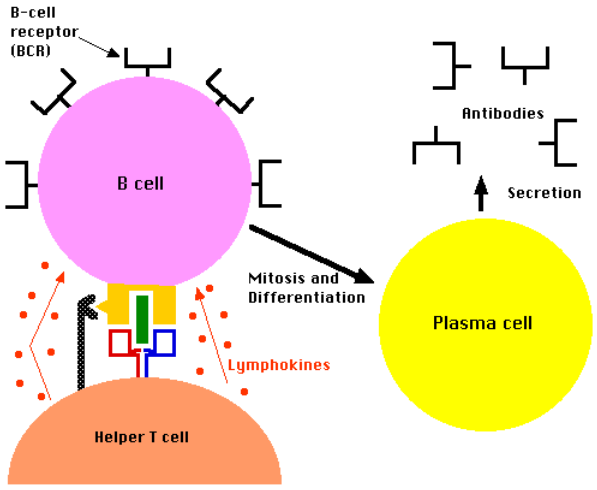


Fig.15.4.3.1 B cell and lymphokines

- BCRs bind intact antigens (like diphtheria toxoid, the protein introduced into your body in the DTP vaccine). These may be
  - soluble molecules present in the extracellular fluid
  - intact molecules that the B cell plucks from the surface of antigen-presenting cells like macrophages and dendritic cells
- The bound antigen molecules are engulfed into the B cell by receptor-mediated endocytosis.
- The antigen is digested into fragments which are then displayed at the cell surface nestled inside a class II histocompatibility molecule.
- Helper T cells specific for this structure (i.e., with complementary TCRs) bind the B cell and secrete lymphokines that:

- stimulate the B cell to enter the cell cycle and develop, by repeated mitosis, into a clone of cells with identical BCRs
- switch from synthesizing their BCRs as integral membrane proteins to a soluble version
- differentiate into plasma cells that secrete these soluble BCRs, which we now call antibodies

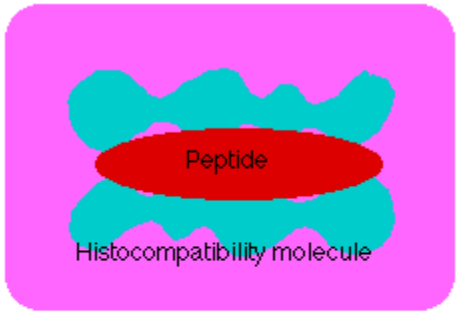
T Cells

The surface of each T cell also displays thousands of identical T cell receptors (TCRs). There are two types of T cells that differ in their TCR:

- alpha/beta (αβ) T cells. Their TCR is a heterodimer of an alpha chain with a beta chain. Each chain has a variable (V) region and a constant (C) region. The V regions each contain 3 hypervariable regions that make up the antigen-binding site.
- gamma/delta (γδ) T cells. Their TCR is also a heterodimer of a gamma chain paired with a delta chain.

The discussion that follows now concerns alpha/beta T cells. Gamma/delta T cells, which are less well understood, are discussed at the end.

The TCR (of alpha/beta T cells) binds a bimolecular complex displayed at the surface of some other cell called an antigen-presenting cell (APC). This complex consists of a fragment of an antigen lying within the groove of a histocompatibility molecule. The complex has been compared to a "hot dog in a bun".



"Hot Dog in a Bun"
Here an antigenic peptide nestled between the alpha helices (blue) exposed at the outer surface of a histocompatibility molecule

Figure 15.4.3.2 T cell structure

Most of the T cells in the body belong to one of two subsets. These are distinguished by the presence on their surface of one or the other of two glycoproteins designated:

- CD4
- CD8

Which of these molecules is present determines what types of cells the T cell can bind to.

- $CD8^+$ T cells bind epitopes that are part of class I histocompatibility molecules. Almost all the cells of the body express class I molecules.
- $CD4^+$ T cells bind epitopes that are part of class II histocompatibility molecules. Only specialized antigen-presenting cells express class II molecules. These include dendritic cells, phagocytic cells like macrophages and B cells.

$CD8^+$ T cells

The best understood $CD8^+$ T cells are cytotoxic T lymphocytes (CTLs). They secrete molecules that destroy the cell to which they have bound.

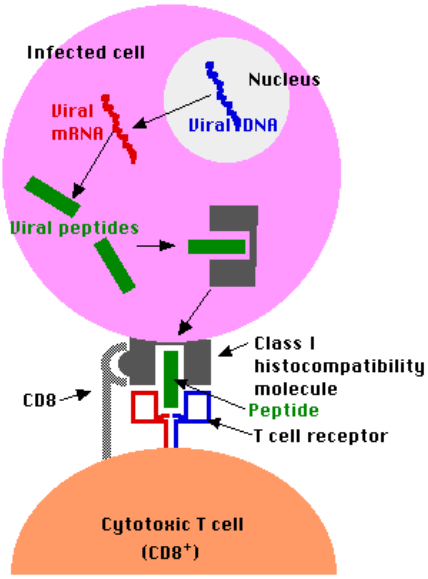


Figure 15.4.3.3 cytotoxic T lymphocytes

This is a very useful function if the target cell is infected with a virus because the cell is usually destroyed before it can release a fresh crop of viruses able to infect other cells. An example will show the beauty and biological efficiency of this mechanism.

Example

Every time you get a virus infection, say influenza (flu), the virus invades certain cells of your body (in this case cells of the respiratory passages). Once inside, the virus subverts the metabolism of the cell to make more virus. This involves synthesizing a number of different macromolecules encoded by the viral genome. In due course, these are assembled into a fresh crop of virus particles that leave the cell (often killing it in the process) and spread to new target cells. Except while in transit from their old homes to their new, the viruses work inside of your cells safe from any antibodies that might be present in blood, lymph, and secretions. But early in the process, infected cells display fragments of the viral proteins in their surface class I molecules. CTLs specific for that antigen will be able to bind to the infected cell and often will be able to destroy it before it can release a fresh crop of viruses.

In general, the role of the $CD8^+$ T cells is to monitor all the cells of the body, ready to destroy any that express foreign antigen fragments in their class I molecules.

$CD4^+$ T cells

$CD4^+$ T cells bind an epitope consisting of an antigen fragment lying in the groove of a class II histocompatibility molecule. $CD4^+$ T cells are essential for both the cell-mediated and antibody-mediated branches of the immune system:

- cell-mediated immunity: These $CD4^+$ cells bind to antigen presented by antigen-presenting cells (APCs) like phagocytic macrophages and dendritic cells. The T cells then release lymphokines that attract other cells to the area. The result is inflammation: the accumulation of cells and molecules that attempt to wall off and destroy the antigenic material (an abscess is one example, the rash following exposure to poison ivy is another).
- antibody-mediated immunity: These $CD4^+$ cells, called helper T cells, bind to antigen presented by B cells. The result is the

development of clones of plasma cells secreting antibodies against the antigenic material.

T cells

AIDS provides a vivid illustration of the importance of $CD4^+$ T cells in immunity. The human immunodeficiency virus (HIV) binds to CD4 molecules and thus is able to invade and infect $CD4^+$ T cells. As the disease progresses, the number of $CD4^+$ T cells declines below its normal level of about 1000 per microliter (µl). A partial explanation for this may be the unceasing efforts of the patient's $CD8^+$ T cells to destroy the infected $CD4^+$ cells. However, it turns out that only a small fraction of the patients $CD4^+$ T cells are infected at any given time. How uninfected $CD4^+$ cells may be induced to commit suicide is discussed in the page on apoptosis.

When the number of $CD4^+$ T cells drops below 400 per microliter, the ability of the patient to mount an immune response declines dangerously. Not only does the patient become hypersusceptible to pathogens that give all of us grief but also to microorganisms, especially viruses and fungi, that normally inhabit our tissues without harming us. These opportunistic infections can be fatal.

Building the T-cell Repertoire

T cells have receptors (TCRs) that bind to antigen fragments nestled in MHC molecules. But all cells express class I MHC molecules containing fragments derived from self proteins. Many cells express class II MHC molecules that also contain self peptides. This presents a risk to the animal of the T cells recognizing these self-peptide/self-MHC complexes and mounting an autoimmune attack against them. Fortunately, this is usually avoided by a process of selection that goes on in the thymus (where all T cells develop).

The process works like this:

- The precursors of T cells like all blood cells are formed in the bone marrow.
- These cells then migrate to the cortex of the thymus. At this time they have neither a complete TCR nor either CD4 or CD8 (thus are called "double-negative" or DN cells).
- In the cortex of the thymus they begin to form a TCR and synthesize both CD4 and CD8 (so now they are "double-positive" or DP cells). The cortical cells of the thymus express a wide variety of small molecules, usually a peptide of 6–8 amino acids derived from body proteins; that is, "self" proteins such as proteins within the cytosol and serum proteins - proteins circulating in the blood and lymph nestled in a histocompatibility molecule (encoded by the MHC).
  - Most of the cells (~97%) will produce a TCR that does not bind to any of the peptide-MHC molecules present on the surface of the cortical cells. Unless they can try again with a new TCR, these cells die by "neglect" (by apoptosis, actually).
  - Those remaining cells whose TCR has bound a peptide antigen presented in class II MHC molecule stop expressing CD8 and become $CD4^+$ T cells. It is these cells that will go on to become
    - Th1 cells in cell-mediated immune responses
    - Th1 helper cells for cytotoxic T lymphocytes (CTLs)
    - Th2 helper cells for B cells
  - Those remaining cells whose TCR has bound a peptide antigen presented in class I MHC molecule stop expressing CD4 and become $CD8^+$ T cells.
  - Both sets of cells are said to have undergone positive selection.
- After positive selection, these cells migrate to the medulla of the thymus.
- There those cells whose TCR binds very strongly to complexes of self-peptide and self-MHC are destroyed (again by apoptosis).
- This process of negative selection is important as it eliminates cells that might otherwise mount an autoimmune attack. It is one of the ways in which tolerance to self antigens is achieved. [Link to discussion of T-cell tolerance.]
- The cells whose TCRs bind antigen at an affinity below the threshold that triggers apoptosis are free to leave the thymus and migrate throughout the immune system (lymph nodes, spleen, etc.)
- It is this population that we depend on to mount immune responses against foreign antigens. A TCR that binds self-peptide/self-MHC with low affinity may well bind a foreign-peptide in self MHC with high affinity.

Gamma/Delta (γδ) T Cells

Gamma/delta T cells differ from their alpha/beta cousins in several ways:

- Their TCR is encoded by different gene segments.
- Their TCR binds to
  - antigens that can be intact proteins (just as antibodies do) as well as a variety of other types of organic molecules (often containing phosphorus atoms).
  - antigens that are not "presented" within class I or class II histocompatibility molecules;
  - antigens that are not presented by "professional" antigen-presenting cells (APCs) like dendritic cells.
- Most of these T cells have neither CD8 nor CD4 on their surface. This makes sense because they have no need to recognize class I and class II histocompatibility molecules.
- Gamma/Delta T cells, like alpha/beta T cells, develop in the thymus. However, they migrate from there into body tissues, especially epithelia (e.g., intestine, skin, lining of the vagina), and don't recirculate between blood and lymph nodes (they represent no more than 5% of the T cells in the blood and are even rarer in lymph nodes). They encounter antigens on the surface of the epithelial cells that surround them rather than relying on the APCs found in lymph nodes.

What is the Function of γδ T cells?

That is still something of a mystery. Situated as they are at the interfaces between the external and internal worlds, they may represent a first line of defense against invading pathogens. Their response does seem to be quicker than that of αβ T cells. Curiously, many of the antigens to which γδ T cells respond are found not only on certain types of invaders (e.g., Mycobacterium tuberculosis, the agent of tuberculosis) but also on host cells that are under attack by pathogens.

Knockout mice that cannot make γδ T cells are slower to heal injuries to their skin. They are also much more susceptible to skin cancers than normal mice. Perhaps immune surveillance is one of the functions of γδ T cells.

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15.4D: Antigen Receptors

This page discusses B cells and T cells, which have unique receptors for specific epitopes that activate immune responses. B cell receptors (BCRs) interact with soluble antigens and produce antibodies, composed of variable and constant regions. There are five classes of antibodies (IgG, IgM, IgA, IgD, IgE), each with distinct roles. It also mentions that abnormal B cells can lead to multiple myeloma by producing a single type of antibody.

Both B cells and T cells have surface receptors for antigen. Each cell has thousands of receptors of a **single specificity**; that is, with a binding site for a particular epitope. T-cell receptors (**TCRs**) enable the cell to bind to and, if additional signals are present, to be activated by and respond to an epitope presented by another cell called the **antigen-presenting cell** or **APC**. B-cell receptors (**BCRs**) enable the cell to bind to and, if additional signals are present, to be activated by and respond to an epitope on molecules of a **soluble antigen**. The response ends with descendants of the B cell secreting vast numbers of a soluble form of its receptors. These are **antibodies**.

Antibodies

Antibodies are glycoproteins. They are built of subunits containing two identical **light chains** (**L** chains), each containing about 200 amino acids and two identical **heavy chains** (**H** chains), which are at least twice as long as light chains. The first 100 or so amino acids at the N-terminal of both H and L chains vary greatly from antibody to antibody. T These are the **variable (V) regions**. Unless members of the same clone (and often not even then), no two B cells are likely to secrete antibodies with the same variable region. The amino acid sequence variability in the V regions is especially pronounced in **3 hypervariable regions**. The tertiary structure of antibodies brings the 3 hypervariable regions of **both** the L and the H chains together. Together they construct the **antigen binding site** against which the epitope fits. For this reason, the hypervariable regions are also called **complementarity determining regions** (CDRs). Only a few different amino acid sequences are found in the C-terminals of H and L chains. These are the **constant (C) regions**.

Humans make two different kinds of C regions for their L chains producing **kappa** (κ) L chains and **lambda** (λ) L chains. They also make five different kinds of C regions for their H chains producing:

- mu (μ) chains (the H chain of **IgM** antibodies)
- **gamma** (γ) chains (**IgG**)
- **alpha** (α) chains (**IgA**)
- **delta** (δ) chains (**IgD**)
- **epsilon** (ε) chains (**IgE**)

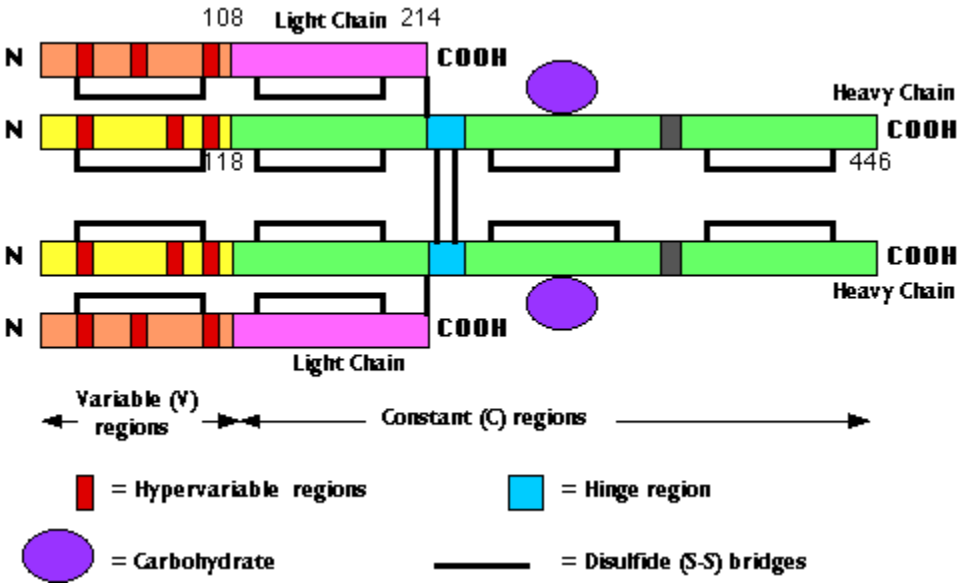


Figure 15.4.4.2 Tertiary structure of L chain courtesy of Dr. D. R. Davies

The images above() represent the folded (tertiary) structure of an **entire L chain** (right side with thin connecting lines) and the **V region** plus the first third of the C region of a **heavy chain** (left side; darker lines). Each circle represents the location in 3D space of an alpha carbon. The filled circles at the top are amino acids in the **hypervariable or complementary determining regions (CDRs)**; they form the site that binds the antigen.

Antibody molecules have two functions to perform:

- recognize and bind to an epitope on an antigen
- trigger a useful response to the antigen

The division of labor is:

- V regions are responsible for epitope recognition.
- C regions are responsible for triggering a useful response

So, V regions finger the culprit; the C regions take action.

If an antibody's H chains (see IgG above), are cut at its hinge region on the N-terminal side of the disulfide bonds holding the H chains together, 3 fragments are produced:

- 2 **Fab** fragments ("fragment antigen-binding") and
- 1 **Fc** fragment ("fragment crystalline" — because the uniformity of this region allows crystals to form while the great diversity of V regions prevents them from forming).

Why 5 kinds of heavy chains? To provide for different effector functions.

The 5 classes of antibodies

Class	H chain	L chain	Subunits	mg/ml	Notes
IgG	gamma	kappa or lambda	H_2L_2	6–13	transferred across placenta; four subclasses: IgG1-4 in humans
IgM	mu	kappa or lambda	$(H_2L_2)_5$	0.5–3	first antibodies to appear after immunization
IgA	alpha	kappa or lambda	$(H_2L_2)_2$	0.6–3	much higher concentrations in secretions; two subclasses
IgD	delta	kappa or lambda	H_2L_2	<0.14	function uncertain
IgE	epsilon	kappa or lambda	H_2L_2	<0.0004	binds to basophils and mast cells sensitizing them for certain allergic reactions

"**mg/ml**" gives the concentration normally found in human serum. The subclasses of IgG and IgA are encoded by different C-region gene segments.

If an antibody-secreting cell becomes cancerous, it will grow into a clone secreting its single class of molecule. The disease is called **multiple myeloma**.

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15.4E: Histocompatibility Molecules

Histocompatibility molecules are glycoproteins expressed at the surface of almost all vertebrate cells. They get their name because they are responsible for the compatibility — or rather the lack of it — of the tissues of genetically different individuals. Monozygotic ("identical") human twins have the same histocompatibility molecules on their cells, and they can accept transplants of tissue from each other. The rest of us have a set of histocompatibility molecules that is probably unique to us. A graft of our tissue into another human will provoke an immune response which, if left unchecked, will end in the rejection of the transplant. So the histocompatibility molecules of one individual act as antigens when introduced into a different individual. In fact, the histocompatibility molecules are often called **histocompatibility antigens** or **transplantation antigens**.

The most rapid and severe rejection of foreign tissue occurs when there is a failure to properly match the donor and recipient for the **major histocompatibility molecules**. There are two categories: **class I** and **class II**.

Class I Histocompatibility Molecules

Class I molecules consist of two polypeptide chains, a long one (on the left) of 346 amino acids — it is called the **heavy chain** — and a short one (on the right) of 99 amino acids. The heavy chain consists of 5 main regions or **domains**:

- three extracellular domains, designated here as **N** (includes the N terminal), **C1**, and **C2**
- a transmembrane domain where the polypeptide chain passes through the plasma membrane of the cell
- a cytoplasmic domain (with the C terminal) within the cytoplasm of the cell

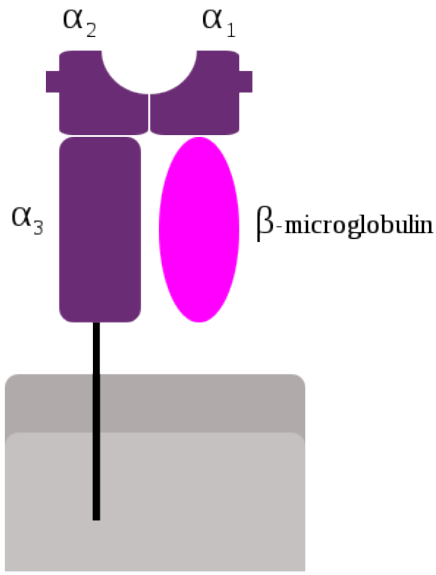


Figure 1: Schematic representation of MHC class I (CC BY 2.5; atropos235).

he above figure shows a protein molecule called **beta-2 microglobulin** ("β2M"). It is not attached to the heavy chain by any covalent bonds, but rather by a number of noncovalent interactions. The dark bars represent disulfide (S-S) bridges linking portions of each domain (except the N domain). However, the bonds in S-S bridges are no longer than any other covalent bond, so if this molecule could be viewed in its actual tertiary (3D) configuration, we would find that the portions of the polypeptide chains containing the linked Cys are actually close together. The outermost domains ("N" and "C1") contain two segments of alpha helix that form two ridges with a groove between them. A small molecule (e.g., a short peptide) is attached noncovalently in the groove between the two alpha helices, rather like a **hot dog in a bun**(See fig. 15.4.5.2)

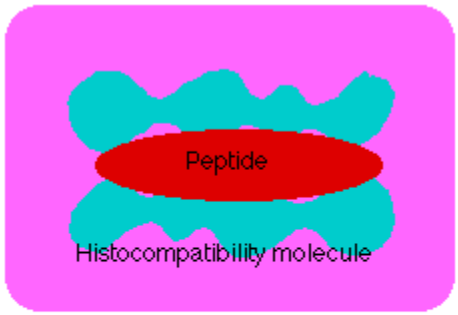
The two objects on the left of the image that look like candelabra represent the short, branched chains of sugars in this glycoprotein. The regions marked "Papain" represent the places on the heavy chain that are attacked by the proteinase papain (and made it possible to release the extracellular domains from the plasma membrane for easier analysis). The image represents the structure of a class I histocompatibility molecule, called H-2K. Almost all the cells of an animal's body (in this case, a mouse)

have thousands of these molecules present in their plasma membrane. These molecules provide tissue identity and serve as major targets in the rejection of transplanted tissue and organs. But tissue rejection is not their natural function. Class I molecules serve to display antigens on the surface of the cell so that they can be "recognized" by **T cells**.

Humans synthesize three different types of class I molecules designated **HLA-A**, **HLA-B**, and **HLA-C**. (HLA stands for **h**uman **l**eukocyte **a**ntigen; because the molecules were first studied on leukocytes). These differ only in their heavy chain, all sharing the same type of beta-2 microglobulin. The genes encoding the different heavy chains are clustered on chromosome 6 in the **major histocompatibility complex** (**MHC**). We inherit a gene for each of the three types of heavy chain from each parent so it is possible, in fact common, to express two allelic versions of each type. Thus a person heterozygous for HLA-A, HLA-B, and HLA-C expresses **six** different class I proteins. These are synthesized and displayed by most of the cells of the body (except those of the central nervous system).

Histocompatibility molecules present antigens to T cells

Although histocompatibility molecules were discovered because of the crucial role they play in graft rejection, clearly evolution did not give vertebrates these molecules for that function. So what is their normal function? The answer: to **display antigens** so that they can be "seen" by T lymphocytes. The antigen receptor on T lymphocytes (or **T cells**, as they are commonly called) "sees" an epitope that is a mosaic of the small molecule in the groove and portions of the alpha helices flanking it.



"Hot Dog in a Bun"
Here an antigenic peptide nestled between the alpha helices (blue) exposed at the outer surface of a histocompatibility molecule

Figure 15.4.5.2 Histocompatibility molecule

The small molecules ("hot dogs") are enormously diverse. They probably represent fragments derived from all the proteins present within the cell. These would include fragments of normal cell constituents, fragments of proteins encoded by intracellular parasites (like viruses), and fragments of proteins encoded by mutated genes in **cancer cells.**

Class II Histocompatibility Molecules

Human class II molecules are designated **HLA-D**, and the genes encoding them are also located in the **major histocompatibility complex** (**MHC**). Class II molecules consist of two transmembrane polypeptides. These interact to form a groove at their outer end which, like class I molecules, always contains a fragment of antigen. But the fragments bound to class II molecules are derived from antigens that the cell has **taken in from its surroundings**. Extracellular molecules are engulfed by endocytosis. The endosomes fuse with lysosomes and their contents are partially digested. The resulting fragments are placed in class II molecules and returned to the cell surface.

Class II molecules, in contrast to class I, are normally expressed on only certain types of cells. These are cells like macrophages and B lymphocytes that specialize in processing and presenting extracellular antigens to **T lymphocytes**. Thus antigen presentation by class II molecules differs from that by class I in two important ways:

- All cells can present antigens with class I molecules, whereas only certain cells can do so with class II.
- The antigen fragments (hot dogs) displayed in class I molecules are generated from macromolecules synthesized within the cell, whereas those displayed in class II molecules have been acquired from outside the cell.

Class I and Class II molecules present antigen fragments to different subsets of T cells

Most of the T cells of the body belong to one of two distinct subsets: **CD4$^+$** or **CD8$^+$**. CD4 and CD8 are surface glycoproteins. Both CD4$^+$ and CD8$^+$ T cells have an antigen receptor (**TCR**) that "sees" a complex hot-dog-in-bun epitope. The CD8 molecules on CD8$^+$ T cells bind to a site found only on class I histocompatibility molecules (shown here as a gray hemisphere). The CD4 molecules on CD4$^+$ T cells bind to a site found only on class II histocompatibility molecules (shown below as a yellow triangle). However, neither type can be **activated** by simply binding its complementary epitope. Additional molecular interactions must take place.

The CD8$^+$ T Cell/Class I Interaction

Because of the need for CD8 to bind to a receptor site found only on class I histocompatibility molecules, CD8$^+$ T cells are only able to respond to antigens presented by class I molecules. Most CD8$^+$ T cells are **cytotoxic T cells** (**CTL**s). They contain the machinery for destroying cells whose class I epitope they recognize.

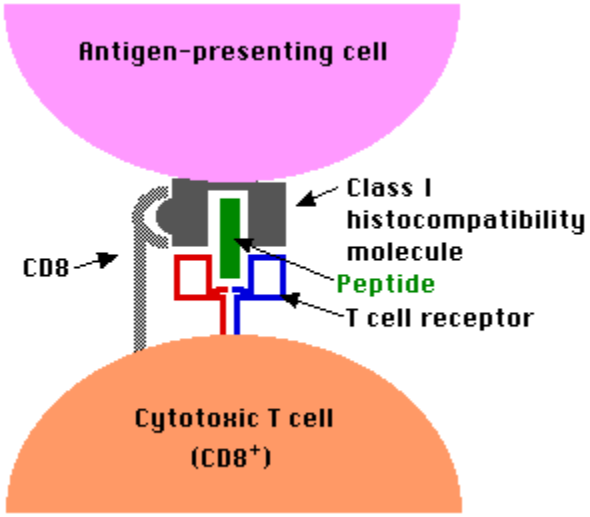


Figure 15.4.5.3 Class I CD8

Example

Every time you get a viral infection, say influenza (flu), the virus invades certain cells of your body. Once inside, the virus subverts the metabolism of the cell to make more virus. This involves synthesizing molecules encoded by the viral genome. In due course, these are assembled into a fresh crop of virus particles that leave the cell (often killing it in the process) and spread to new cells. Except while in transit from their old home to their new, the virus works inside cells safe from any antibodies. But early in their intracellular life, infected cells display fragments of the viral proteins being synthesized in the cytoplasm in their surface class I molecules. Any cytotoxic T cells specific for that antigen will bind to the infected cell and often will be able to destroy it before it can release a fresh crop of virus.

The bottom line: the function of the body's CD8$^+$ T cells is to monitor all the cells of the body ready to destroy any that express foreign antigen fragments in their class I molecules.

The CD4$^+$ T Cell/Class II Interaction

The CD4 molecules expressed on the surface of CD4$^+$ T cells enable them to bind to cells presenting antigen fragments in class II molecules but not in class I. Only certain types of cells, those specialized for taking up antigen from extracellular fluids, express class II molecules. Among the most important of these are dendritic cells, macrophages (phagocytic cells that develop from monocytes that have migrated from the blood to the tissues), and B lymphocytes ("B cells") that take up exogenous antigen by receptor-mediated endocytosis.

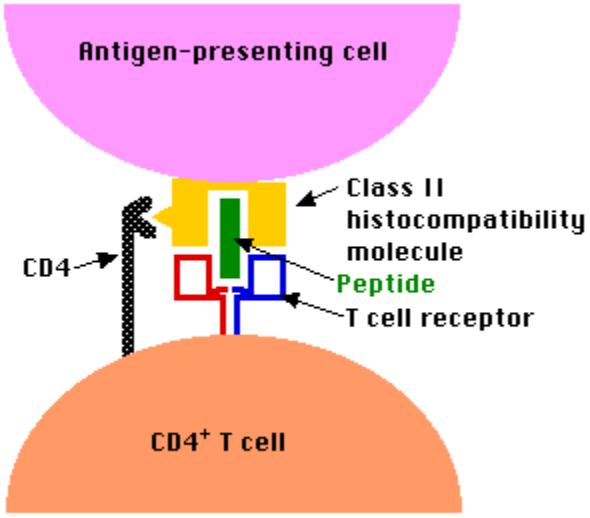


Figure 15.4.5.4 Class II CD4

So CD4$^+$ T cells see antigen derived from extracellular fluids and processed by specialized antigen-presenting cells. To respond to an antigen, a CD4$^+$ T cell must have a **T cell receptor** (**TCR**) able to recognize (bind to) a complex epitope comprising an antigenic fragment displayed by a class II molecule, bind a site on the class II molecule (shown above as a yellow triangle) with its CD4, and bind to costimulatory molecules on the antigen-presenting cell. If these conditions are met, the T cell becomes activated. Activated T cells enter the cell cycle leading to the growth of a clone of identical T cells and begin to secrete lymphokines.

Lymphokines activate and recruit other cells (e.g., mast cells) to the region producing inflammation (e.g., to cope with a bacterial infection). They also activate B cells enabling them to develop into a clone of antibody-secreting cells. The CD4$^+$ T cells that activate B cells are called **Helper T cells.**

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15.4F: Antigen Receptor Diversity

The human genome is presently estimated to contain 20–25 thousand genes. The number of T-cell receptors for antigen (**TCR**s) that we make is estimated at 2.5 x 10^7; the number of different kinds of antibody molecules (**BCR**s) is probably about the same.

How could 2.5 x 10^4 genes encode 2.5 x 10^7 different TCRs and the same number of different BCRs?

The answer: each receptor chain

- **heavy (H)** plus **kappa (κ)** or **lambda (λ)** chains for BCRs;
- **alpha (α)** and **beta (β)** or **gamma (γ)** and **delta (δ)** chains for TCRs)

is encoded by several different **gene segments**. The genome contains a pool of gene segments for each type of chain. Random assortment of these segments makes the largest contribution to receptor diversity.

B cells

Gene segment usage for BCRs

For the **heavy** (**H**) **chains** of BCRs (antibodies), the gene segments are:

- 51 **V_H** segments. Each of these encodes most of the N-terminal of the antibody, including the first two (but not the third) **hypervariable or complementarity determining region** (**CDR**).
- 27 **D_H** (="diversity") gene segments. These encode part of the third CDR ("CDR3").
- 6 **J_H** (="joining") gene segments. These encode the remainder of the V region of the BCR (including the remainder of CDR3).
- 9 **C_H** gene segments. These encode the C region of the BCR (and the antibody derived from it). The C gene segments are
  - 1 **mu (μ)**; encodes the C region of **IgM**
  - 1 **delta (δ)** for **IgD**
  - 4 **gamma (γ)** gene segments for the four types of **IgG**
  - 1 **epsilon (ε)** for **IgE**
  - 2 **alpha (α)** gene segments for the two types of **IgA**

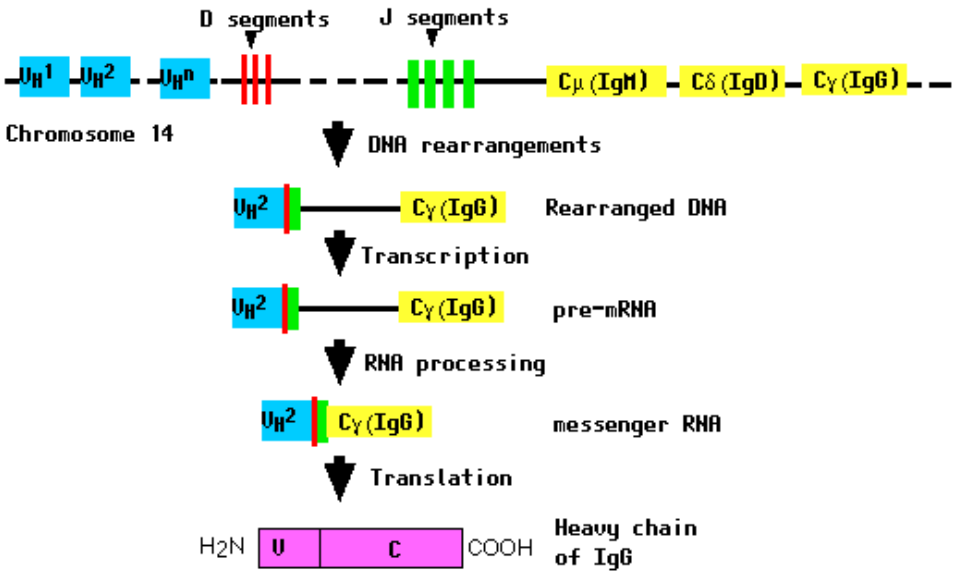


Figure 15.4.6.1 VH Locus

All of these gene segments are clustered in a complex locus on **chromosome 14.** During the differentiation of the B cell (and long before any encounter with an antigen), the DNA in this locus is cut and recombined to make an intact gene for the heavy chain. This gene can then be **transcribed** into **pre-mRNA**, which is then processed to form the mRNA that will be **translated** into the heavy (H) polypeptide chain.

V(D)J Joining

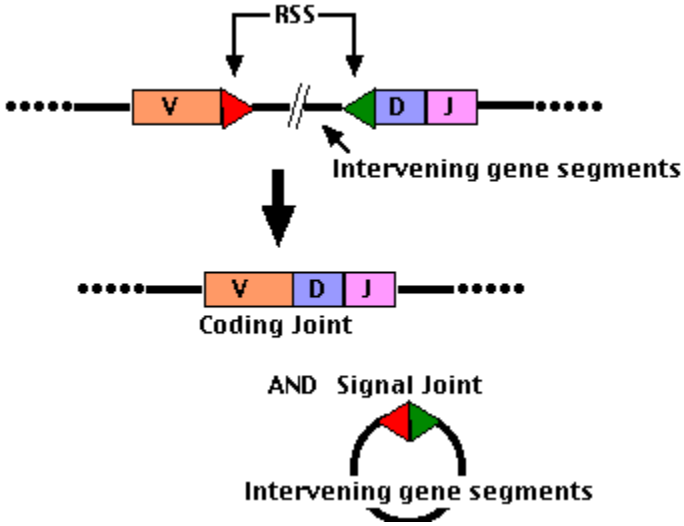


Figure 15.4.6.2 V-D-J segment

- Each gene segment (V, D, and J) has an adjacent **Recombination Signal Sequence** (**RSS**)
  - at the 3' end of each V segment
  - at both ends of each D segment
  - at the 3' end of each J segment
- These are recognized by two proteins encoded by two **R**ecombination **A**ctivating **G**enes
  - ***RAG-1***
  - ***RAG-2***
- The RAG-1 and RAG-2 proteins cut through both strands of DNA at the RSS forming double-stranded breaks (DSBs).
- Then the regular machinery for repairing DSBs (by **nonhomologous end-joining**) swings into action.
- The cut ends are stitched together (ligated) to form:
  - a **coding joint** (D-J or V-DJ for heavy chains; V-J for light chains)
  - a **signal joint** (usually a loop of DNA deleting all the intervening DNA initially present between the 2 gene segments chosen).
- D-J joining occurs first; then the combined DJ segment (still attached to the cluster of constant region gene segments) is joined to a V segment (as shown in the figure).
- The V gene segment chosen may be thousands of base pairs away from the D-J segment so the chromosome must be drawn into a loop to bring the two together. The loop is stabilized by
  - a protein designated **CTCF** ("CCCTC binding factor"; named for the nucleotide sequence to which it binds). The CTCF at the D-J site on the DNA forms a dimer with the CTCF at the V site on the DNA binding the two regions together.
  - **cohesin** — the same protein complex that holds sister chromatids together during mitosis and meiosis.
- In the process, the cluster of gene segments moves from the periphery of the nucleus (a region of inactive genes) to the center of the nucleus (a region of active gene transcription).

SCID

Some cases of **severe combined immunodeficiency** in humans (**SCID**) are caused by defects in V(D)J joining.

- One version is caused by mutations in both copies of either *RAG1* or *RAG2*.
- Another is caused by mutations in a gene needed for nonhomologous end-joining. (No coding joint is formed even though a signal joint forms normally.)

If the 51 **V_H**, 27 **D_H**, and 6 **J_H** gene segments were assembled randomly (they probably are not), that would provide a minimum of 8.3 x 10^3 different possible combinations. But the possibilities of antibody V region diversity turn out to be greater than that. The recombination process is not precise.

- The exact points of splicing between V_H and D_H and between D_H and $J_H$can vary over several nucleotides
- Extra nucleotides, called **N regions**, can also be inserted at these joints.
- All of these add greatly to the diversity of CDR3.

Light chains

Once the H chain gene is assembled, transcribed, and translated, the resulting H chain can pair with an L chain that is itself the product of a similar recombination process occurring

- on **chromosome 2** for **kappa** gene segments
- on **chromosome 22** for **lambda** gene segments

Antibodies (BCRs)	Gene Segments	Combinations
V_κ	40	
J_κ	5	200 κ chains
V_λ	31	
J_λ	4	124 λ chains
V_H	51	
D_H	25	
J_H	6	7,650 H chains
Any H chain with any L chain (324)		2.5 x 10^6

As the table shows, this lays the foundation for a potential B-cell repertoire of 2.5 x 10^6 different antibody V regions. But the true number is probably virtually limitless because of variation in the exact splice point and the introduction of N nucleotides both of which increase the diversity of CDR3.

Diversity comes at a price

The combining of V, D, and J gene segments coupled with the random incorporation of extra nucleotides (N regions) at the joints, creates enormous coding variability. It also creates a high risk (two times out of three) of introducing a frameshift so that the codons for the rest of the V region encode nonsense. Although many B cells are wasted, the odds are not quite as bad as they seem. If the B cell fails to make a functional product from the cluster of gene segments on one of its chromosomes, it can turn to the gene segments on its homolog and try again. If it fails both times to make a functional kappa L chains, it still has two tries at making a functional lambda L chain.

Somatic Hypermutation (SHM) and Antibody Diversity

The diversifying mechanisms described above take place **before** the B cell encounters antigen. **After** a B cell encounters antigen, it may begin mitosis, growing into a clone of cells synthesizing the same BCR (and, eventually, secreting antibodies with the same binding site). Point mutations can occur while this is going on. Some of these may generate a binding site with **increased affinity** for its epitope. These are favorable mutations, and the "subclone" in which they occur tends to be favored and may replace the ancestral clone. The result is **affinity maturation** — the production of antibodies of ever-increasing affinity for the antigen.

Class Switch Recombination (CSR)

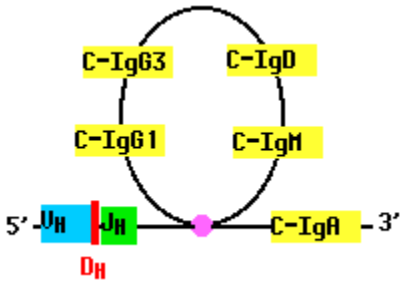


Figure 15.4.6.3 Class switch

As B cells grow into a clone in response to antigen, they may rearrange their DNA once again. For example, a B cell that has assembled a complete gene for the H chain of IgM (μ), may cut the gene on the 3´ side of the assembled V-region segments and move the assembly to the 5´ side of another of its C_H gene segments. Now the cell begins to make a different class of antibody, such as IgG or IgA. But the antigen specificity of the antibody remains the same because the N-terminal of the H chain remains unchanged (as does the entire L chain).

Class switch recombination enables the body to produce antibodies with different **effector functions**; that is, different means of dealing with the same antigen. The ability of a B cell to switch C_H gene segments depends on its receiving help from helper T cells.

T Cells

Alpha/beta (αβ) T cells

The most abundant T cells in the **blood** express a receptor for antigen (**TCR**) that is a heterodimer of two chains designated alpha (α) and beta (β). Each of these is encoded by a gene assembled from V, D, J, and C gene segments. Like BCRs, there are multiple variants of these gene segments arranged in clusters:

- **alpha** chain gene segments on chromosome 14
- **beta** chain gene segments on chromosome 7

T cell receptors (TCRs)	Gene segments	Combinations
Vα	50	
Jα	50	2.5×10^3 alpha chains
Vβ	20	
Jβ	13	
Dβ	2	520 beta chains
Any alpha with any beta chain		1.3×10^6

And like B cells, the greatest diversity in the receptors of αβ T cells occurs in the third complementarity determining region (**CDR3**) of the alpha and beta chains because of the junctional diversity between the V, J, and D segments and the addition of **N region** nucleotides. However, T cells do not seem to use somatic mutation to increase receptor diversity. Actual measurements of the repertoire in humans reveals a figure of about 2.5×10^7.

Gamma/delta (γδ) T cells

The TCR repertoire of γδ T cells seems much smaller than that of their αβ cousins. The gamma chain gene segments are clustered on chromosome 7. The delta chain gene segments are clustered on chromosome 14 (within the alpha chain cluster).

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15.4G: Anatomy of the Immune System

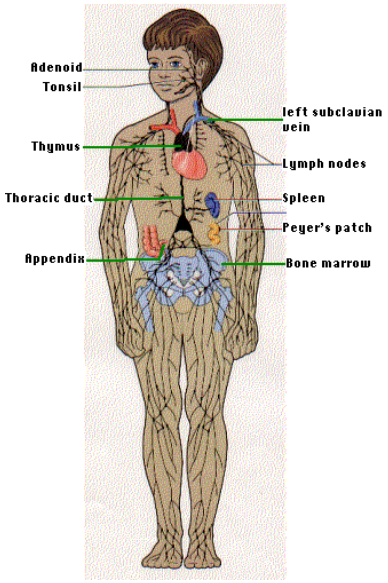


Figure 15.4.7.1 Immune system

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15.4H: T Helper cells

T cells are T lymphocytes that belong to the **$CD4^+$** subset. These cells have a number of direct functions, but they get their name from the help they provide to other types of effector cells, such as B cells and cytotoxic T lymphocytes (CTLs). The help consists of secreted cytokines that stimulate the helped cells.

Types of Helper T Cells

Four kinds have been identified:

- **Th1**
  - These participate in both **cell-mediated immunity** and antibody-mediated immunity. They are essential for controlling such **intracellular** pathogens as viruses and certain bacteria, e.g., Listeria and Mycobacterium tuberculosis (the bacillus that causes TB). They provide cytokine-mediated "help" to cytotoxic T lymphocytes — perhaps the body's most potent weapon against intracellular pathogens.
- **Th2**
  - These provide help for B cells and are essential for the production of IgE antibodies and assist in the production of some subclasses of IgG as well. Antibodies are needed to control **extracellular** pathogens (which unlike intracellular parasites are exposed to antibodies in blood and other body fluids).
- **Tfh**
  - These also provide help to B cells enabling them to develop into antibody-secreting plasma cells. This occurs in nests of lymphoid cells called follicles — in the lymph nodes. The most abundant helper T cells there are B-cell helpers called **follicular helper T** (Tfh) cells.
- **Th17**
  - These protect surfaces (e.g., skin, lining of the intestine) against extracellular bacteria.

In addition, there is another related subset that dampens rather than promotes immune responses. These cells, designated **Treg**, are discussed on another page. Link to it.

Origin of Helper T Cells

Like all T cells, Th cells arise in the **thymus**.

- When they acquire CD4, they are called **pre-Th** cells.
- When they are presented with both an antigen and appropriate cytokines,they begin to proliferate and become activated.
- It is the nature of the stimulation — the type of antigen-presenting cell and cytokine(s) — that determines which path they enter.

Th1 Cells

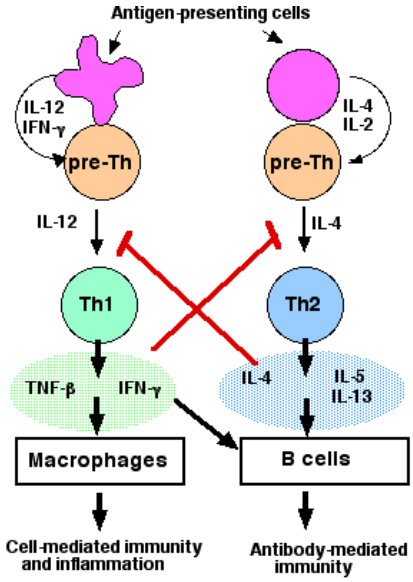


Figure 15.4.8.1 Th1 and Th2 cells

Th1 cells are produced when dendritic cells and pre-Th cells form an immunological synapse in which the dendritic cell presents antigen to the T cell's receptor for antigen (TCR), and secretes interleukin 12 (**IL-12**) as well as IFN-γ. The paracrine stimulation by these cytokines causes the Th1 cells to secrete their own lymphokines:

- interferon-gamma (**IFN-γ**)
- tumor-necrosis factor-beta (**TNF-β**) (also known as **lymphotoxin**)

These stimulate macrophages to kill the bacteria they have engulfed, recruit other leukocytes to the site producing inflammation, act on B cells to promote antibody class switching, and help cytotoxic T cells (CTL) do their work and probably help convert some of them to memory cells.

Th2 Cells

Th2 cells are produced when antigen-presenting cells (APCs) present antigen (e.g., on parasitic helminth worms or certain allergens) to the T cell's receptor for antigen (TCR) along with

- the costimulatory molecule B7 (CD80 & 86)
- the paracrine stimulants interleukin 4 (IL-4) and interleukin 2 (IL-2).

The identity of the APCs for Th2 responses is still uncertain. Some research indicates that basophils are the APCs, but other research questions this role.

The major lymphokines secreted by Th2 cells are

- **interleukin 4** (**IL-4**). This
  - stimulates class-switching in B cells and promotes their synthesis of IgE antibodies.
  - acts as a positive-feedback device promoting more pre-Th cells to enter the Th2 pathway.
  - blocks the IFN-γ receptors from entering the immunological synapse on pre-Th cells thus inhibiting them from entering the Th1 path (shown in red).
- **Interleukin 13** (**IL-13**). This also promotes the synthesis of **IgE** antibodies as well as recruiting and activating basophils.
- **Interleukin 5** (**IL-5**). Attracts and activates eosinophils.

Two transcription factors have been found that play a critical role in the choice between becoming a Th1 or a Th2 cell.

- **T-bet** for Th1 cells

- **GATA3** for Th2 cells

T-bet produces Th1 cells by

- turning on the genes needed for Th1 function (e.g., for IFN-γ)
- blocking the activity of GATA3.

Mice whose genes for T-bet have been "knocked-out" lack Th1 cells and have elevated numbers of Th2 cells (making them susceptible to such Th2-mediated disorders as asthma).

GATA3 produces Th2 cells by

- turning on the genes needed for Th2 function (e.g., for IL-5)
- blocking the activity of T-bet

Reciprocal inhibition of Th1 and Th2 cells.

The antigenic stimulus that sends pre-Th cells down one path or the other also sets the stage for reinforcing the response.

A Th1 response inhibits the Th2 path in two ways:

- **IFN-γ** (shown above in red) and **IL-12** inhibit the formation of Th2 cells
- **IFN-γ** also inhibits class-switching in B cells

A Th2 response inhibits the Th1 path:

- **IL-4** suppresses Th1 formation (shown above in red)
- significance for public health: infection by helminths — common in the tropics — increases one's risk of viral and bacterial diseases, and in laboratory mice has been shown to enhance viral infections

Negative feedback of Th1 and Th2 cell formation

There is also evidence that **late** in the immune response, negative feedback mechanisms come into play to dampen the response.

- IL-4 kills (by apoptosis) the precursors of the dendritic cells that induce the Th2 path and thus further production of IL-4.
- **IFN-γ** may eventually turn off the Th1 response that produced it.

Th1 and Th2 cells have different chemokine receptors.

Chemokines are cytokines that are chemotactic for (attract) leukocytes. The members of one group, who share a pair of adjacent cysteine (C) residues near their N-terminal, are designated CC chemokines. Chemokines bind to receptors on the responding leukocyte. The receptors are transmembrane proteins with the chemokine binding site exposed at the surface of the plasma membrane. CC chemokine receptors are designated **CCR**.

With their different functions, we might expect that Th1 cells and Th2 cells would respond differently to chemokines. And so they do.

Th1 cells express the chemokine receptor **CCR5** (but not CCR3).

Th2 cells express the chemokine receptor **CCR3** (but not CCR5).

CCR3

One chemokine that binds to CCR3 is called **eotaxin**. It is secreted by epithelial cells and phagocytic cells in regions where allergic reactions are occurring.

CCR3 is found on all cells implicated in allergic responses (e.g., asthma). They are:

- Th2 cells
- eosinophils
- basophils

CCR5

CCR5 is found on

- Th1 cells, especially those in the lymphoid tissue of the intestine
- macrophages

CCR5 also acts — along with the CD4 molecule — as a coreceptor for **HIV-1**, the retrovirus that causes **AIDS**. This fact may explain

- why destruction of the lymphoid tissue of the intestine occurs soon after HIV infection;
- why certain HIV-infected men
  - with inherited mutations preventing the expression of CCR5 or
  - who produce high levels of the natural ligand for CCR5 (a chemokine designated CCL3L1, which presumably competes with HIV for access to CCR5)

  can tolerate their infection for long periods without progressing to a full-blown case of AIDS;
- the collapse of cell-mediated immunity in the late stages of AIDS

Example

One striking illustration: an AIDS patient with leukemia was given a bone marrow transplant from a donor whose cells expressed a nonfunctional version of CCR5. Two years later, the patient was not only cured of his leukemia but of AIDS as well.

Another: **Gene therapy** in which samples of a patient's $CD4^+$ T cells were treated in vitro so that their *CCR5* gene became nonfunctiona. Expanded in culture and then returned to the donor, five (of six) patients had their $CD4^+$ T cell counts rebound.

Tfh Cells

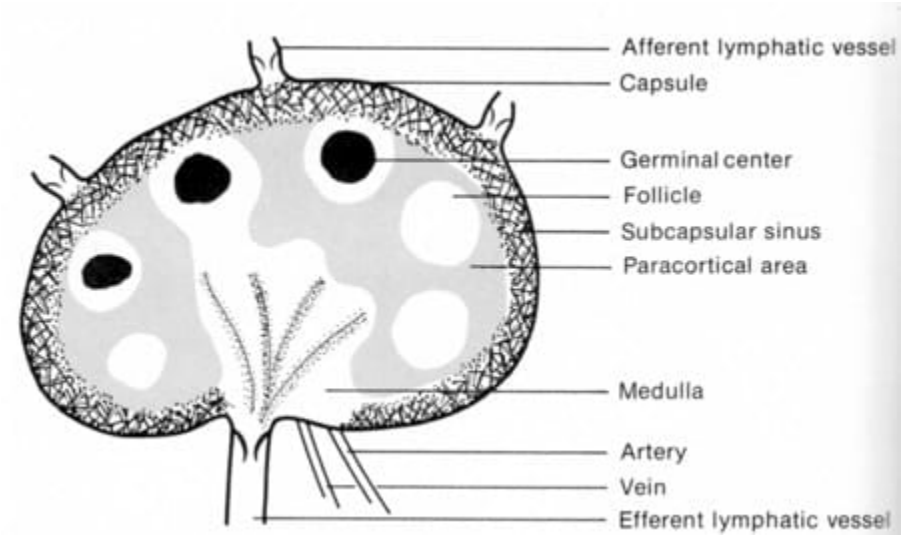


Figure 15.4.8.2 Lymph node

Follicular helper T cells (Tfh) are $CD4^+$ helper T cells found in nests of B cells called follicles — in the lymph nodes. When exposed (in the paracortical area of the lymph node) to cells presenting antigen to them, e.g., dendritic cells and a cocktail of cytokines they migrate into the follicles. The combined stimuli of antigen binding to their TCR and exposure to cytokines activate a transcription factor called Bcl-6 (first identified in a B-cell lymphoma). Bcl-6 turns on a collection of genes which, among other things, cause

the Tfh cells to form an immunological synapse with those B cells expressing the antigen fragments in class II histocompatibility molecules that match their TCR.

Several other pairs of ligands and their receptors stabilize the synapse, including the interaction between CD28 on the Tfh cell and its ligand, B7, on the B cell. These binding interactions stimulate the B cell to

- undergo class switching with the synthesis of other antibody classes (except IgE)
- undergo affinity maturation
- form antibody-secreting plasma cells and memory B cells

This intense activity within the follicle forms a germinal center. It is not yet certain whether Tfh cells represent a distinct class of Th cells or are simply a further stage in the maturation of Th1, or Th2, or Th17 cells.

Th17 Cells

Th17 cells are a recently-identified subset of $CD4^+$ T helper cells. They are found at the interfaces between the external environment and the internal environment, e.g., skin and lining of the GI tract. They probably start out like other "naive" Th cells, but when exposed to

- cells presenting antigen to them, e.g., dendritic cells
- several cytokines notably transforming growth factor-beta **TGF-β** and IL-6

they enter a pathway distinct from that of Th1, Th2, and Tfh cells. The combined stimuli of antigen binding to the TCR and exposure to the cytokinesactivate a nuclear retinoid receptor designated RORγt. This is a transcription factor that turns on a collection of genes which, among other things, leads to

- the synthesis and secretion of IL-17 (giving the cells their name)
- increased synthesis of the plasma membrane receptor for the interleukin IL-23. Interaction of IL-23 (perhaps secreted from nearby dendritic cells) with the receptor drives the rapid proliferation of the Th17 cells

Situated in the skin and the lining of the GI tract, Th17 cells are positioned to attack fungi and bacteria at those locations. They do this by secreting **defensins** and recruiting scavenging cells, especially neutrophils, to the site. The result: clearing away of the invaders with accompanying inflammation.

But inflammation is a double-edged sword. So it is not surprising that Th17 cells have been implicated as potent effectors of such damaging inflammatory disorders as

- Crohn's disease (an inflammation of the small intestine)
- Ulcerative colitis (inflammation of the large intestine)
- Psoriasis (inflammation of the skin)
- An animal model (in mice) of multiple sclerosis
- Rheumatoid arthritis

Summary Table

Type	Cytokine Stimulus	Master Transcription Factor	Effector Cytokine(s)	Main Target Cells	Effector Targets/Functions	Pathological Effects
Th1		T-bet	IFN-γ & TNF-β	Macrophages, dendritic cells	Intracellular pathogens	Autoimmunity; cell-mediated allergies
Th2	IL-4	GATA3		Eosinophils, basophils, B cells	Various helminths	Asthma and IgE-mediated allergies
Tfh		Bcl-6		B cells	Class Switch Recombination and Affinity Maturation of antibodies	Autoimmune diseases?

Type	Cytokine Stimulus	Master Transcription Factor	Effector Cytokine(s)	Main Target Cells	Effector Targets/Functions	Pathological Effects
Th17	TGF-β **plus** IL-6 Inhibited by retinoic acid	RORγt		Neutrophils	Extracellular bacteria and fungi mediates inflammation	Autoimmune diseases
pTreg	TGF-β **minus** IL-6 Stimulated by retinoic acid and **IL-2**	Foxp3		all the other types of T cells	Immunosuppression; anti-inflammatory	None?

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15.4I: Cytotoxic T lymphocytes (CTL)

This page discusses cytotoxic T lymphocytes (CTLs), primarily CD8+ T cells, which identify and kill infected or cancerous cells through αβ T-cell receptors and class I MHC molecules. Upon activation, CTLs expand and differentiate into effector cells with helper T cell assistance, utilizing perforin and granzymes to trigger apoptosis in targets.

Cytotoxic T lymphocytes are lymphocytes that kill other ("target") cells.

Targets may include:

- virus-infected cells (e.g., HIV-infected $CD4^+$ T cells)
- cells infected with intracellular bacterial or protozoal parasites
- allografts such as transplanted kidney, heart, lungs, etc.
- cancer cells. (The tumor-infiltrating lymphocytes, **TIL**, that have shown some promise in cancer therapy contain CTLs.)

There is also evidence that CTL are active in some autoimmune disorders, e.g. help destroy the beta cells of the islets of Langerhans, leading to Type I diabetes mellitus.

Properties of CTLs

Most of them

- belong to the $CD8^+$ subset of T cells;
- use the αβ T-cell receptor for antigen (TCR)
- thus recognize antigens nestled in the groove of class I histocompatibility (MHC) molecules.
- If they encounter (on a dendritic cell) the antigen/MHC for which their TCR is specific, they
  - enter the cell cycle and go through several rounds of mitosis ("clonal expansion") followed by
  - differentiation into effector ("killer") cells. Their differentiation includes forming a large number of modified lysosomes stuffed with proteins: perforin and several types of granzyme. They are helped in these activities by helper T cells that secrete stimulatory cytokines like IL-21.
  - Most of these CTLs will die (of apoptosis) when they have done their job, but some (especially those that have received "help" from helper T cells) will become **memory cells** — long-lived cells poised to respond to the antigen if it should reappear.

An example will show the beauty and biological efficiency of CTLs.

Every time you get a virus infection, say influenza (flu), the virus invades certain cells of your body (in this case cells of the respiratory passages). Once inside, the virus subverts the metabolism of the cell to make more virus. This involves synthesizing a number of different macromolecules encoded by the viral genome. In due course, these are assembled into a fresh crop of virus particles that leave the cell (often killing it in the process) and spread to new target cells. Except while in transit from their old homes to their new, the viruses work inside of your cells safe from any antibodies that might be present in blood, lymph, and secretions. But early in the process, infected cells display fragments of the viral proteins in their surface class I molecules. CTLs specific for that antigen will be able to bind to the infected cell and often will be able to destroy it before it can release a fresh crop of viruses.

In general, the role of the $CD8^+$ T cells is to monitor all the cells of the body, ready to destroy any that express foreign antigen fragments in their class I molecules. Some $CD4^+$ T cells can develop into CTLs, but they can attack only those cell types (e.g. B cells, macrophages, dendritic cells) that express class II MHC molecules. Virtually every cell in the body expresses class I MHC molecules, so $CD8^+$ CTLs are not limited in the targets they can attack.

Mechanisms of Killing

There are two different types of mechanisms.

Perforin/Granzyme Killing

CTLs have cytoplasmic granules that contain the proteins **perforin** and **granzymes**. When the CTL binds to its target, the contents of the granules are discharged by exocytosis. A dozen or more perforin molecules insert themselves into the plasma membrane of target cells forming a pore that enables **granzymes** to enter the cell. Granzymes are serine proteases. The two most abundant ones are

- Granzyme A. Once inside the cell, it enters the mitochondria and cleaves a subunit of complex I (the NADH dehydrogenase) of the electron transport chain producing reactive oxygen species (ROS) that kill the cell.
- Granzyme B. Once inside the cell, it proceeds to cleave the precursors of **caspases** thus activating them to cause the cell to self-destruct by apoptosis.

Both in structure and function, the interaction of CTL and its target resembles the synapses of the nervous system. The two cells are attached tightly at a small patch of plasma membrane. Special adhesion molecules hold them together. The granules are discharged only at that small portion of the plasma membrane (like the neurotransmitters released at a synapse).

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15.4J: Cell-Mediated Immunity

This page explains the two types of immunity in the human body: antibody-mediated (humoral) immunity, where antibodies interact with antigens in body fluids, and cell-mediated immunity, which involves T cells responding to antigens on cells. It highlights instances such as delayed-type hypersensitivity and contact sensitivity. It also notes that some pathogens evade antibodies by invading cells, but activated T cells can enhance macrophage activity to combat these threats.

The human body can respond to antigen in many different ways. These fall into two major categories:

- **antibody-mediated immunity**. Antibodies — dissolved in blood, lymph, and other body fluids — bind the antigen and trigger a response to it. (This form of immunity is also called **humoral immunity**.)
- **cell-mediated immunity** (**CMI**). T cells (lymphocytes) bind to the surface of other cells that display the antigen and trigger a response. The response may involve
  - other lymphocytes
  - any of the other white blood cells (leukocytes)

Examples of Cell-Mediated Immunity

Delayed-Type Hypersensitivity (DTH): the tuberculin test

Many states in the United States require that professors and teachers (among others) be checked periodically for tuberculosis. This chronic disease, caused by **Mycobacterium tuberculosis**, evokes an immune response that, unfortunately, does not cure the patient, but does provide an inexpensive test for the disease called the **tuberculin test** (or Mantoux test). A tiny amount of protein, extracted from the bacteria, is injected into the skin. If the subject is currently infected, **or has ever been infected**, with the bacteria, a positive test results. In 24 hours or so, a hard, red nodule develops at the site of the injection. This nodule is densely packed with lymphocytes and macrophages.

In Europe, most people produce a positive tuberculin reaction, not because they have had the infection, but because earlier they had been vaccinated against tuberculosis with a preparation of a related (but harmless) bacterium called **BCG**.

The response to tuberculin is called "delayed" because of the time it takes to occur (in contrast to the "immediate" responses characteristic of many antibody-mediated sensitivities like an allergic response to a bee sting).

DTH is a cell-mediated response (in fact, anti-tuberculin antibodies are rarely found in tuberculin-positive people). The T cells responsible for DTH are members of the $CD4^+$ subset.

Contact Sensitivity

Many people develop rashes on their skin following contact with certain chemicals. Nickel, certain dyes, and the active ingredient of the poison ivy plant are common examples. The response takes some 24 hours to occur, and like DTH, is triggered by $CD4^+$ T cells. The actual antigen is probably created by the binding of the chemical to proteins in the skin. After the antigen is engulfed by dendritic cells in the skin, they migrate to nearby lymph nodes where they present fragments of the antigen to $CD4^+$ T cells. The activated T cells migrate from the lymph nodes to the skin to elicit the inflammatory response.

Killing intracellular parasites

Some human pathogens avoid exposure to antibodies by taking up residence within cells. These include all viruses (discussed in the next section), and some bacteria such as

- the bacterium that causes **Legionnaires's disease**
- Listeria monocytogenes, that humans sometimes acquire from contaminated food and even some protozoans.

These microorganisms are engulfed by phagocytic cells, like macrophages, but evade the normal intracellular mechanisms that

should destroy them. However, the macrophages can present fragments of antigens derived from these parasites. These are displayed in the class II histocompatibility molecules of the macrophages. **$CD4^+$ T cells** responding to these epitopes release lymphokines that stimulate the macrophages sufficiently that they can now begin to destroy the organisms.

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15.4K: Organ Transplants

New parts for old

Many organs and tissues are now routinely transplanted from one human to another. Except for the rare cases where the donor and recipient are monozygotic ("identical") twins, such grafts are called **allografts**.

- **kidney**
  - Living donors can be used because they have two kidneys and can get along with only one.
- **heart**
  - For patients with failing hearts (often because of inherited defects). Only cadavers can be used as donors.
- **lungs**
  - Usually transplanted along with a heart. Some attempts have been made with portions of lungs from living donors.
- **pancreas**
  - For people with **Type 1 diabetes mellitus**.
- **liver**
  - For irreversible liver failure (e.g., from toxins, hepatitis B infection).
- **skin**
  - For burns; usually taken from elsewhere on the patient's own body.
- **cornea**
  - To restore sight; taken from cadavers.
- **blood**
  - To temporarily restore blood volume.
- **bone marrow**
  - As a source of blood ("hematopoietic") stem cells to repopulate the patient's own marrow that is
    - congenitally deficient in its ability to make one or more kinds of blood cells — Example: severe combined immunodeficiency (SCID)
    - has been destroyed by cancer therapy.
- **cord blood**
  - Blood drained (through the umbilical cord) from the placenta of newborn infants. A convenient source of blood stem cells.
- **ovary**
  - Has restored fertility and produced healthy babies but so far only when donor and recipient were monozygotic (identical) twins.

The Problems

- **Graft rejection**
  - The patient's immune system "sees" an allograft as foreign (antigenic) and mounts an immune response against it.
- **Graft-versus-host disease** (**GVHD**)
  - T cells in the graft "see" the tissues of the recipient as foreign antigens and mount an immune attack against them. This a particularly serious problem with grafts of bone marrow because of the many T cells in it.
- **Infections**
  - Attempts to suppress the immune response to avoid graft rejection and GVHD weaken the ability of the body to combat infectious agents (bacteria, viruses, fungi).
  - More rarely, the donated organ may be infected and transmit the agent to the recipient. Tuberculosis, rabies, syphilis, hepatitis B, HIV-1, and several other diseases have been transmitted in this way. Potential organ donors are now routinely tested for evidence of infection by HIV-1 and -2, HTLV-1 and -2, hepatitis B and C (HBV, HCV), human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) as well as by Treponema pallidum (syphilis).
- **Cancer**
  - Suppressing the host's immune responses also increases the risk of cancer.

Coping with the Immunological Problems

- Use the patient's own tissue when possible (skin, bone marrow, blood vessels).
- Use tissue from an "identical" (monozygotic) twin in the very rare cases that one is available. Being genetically identical, the recipient sees the transplant as "self", not as foreign, and does not mount an attack against it. The first successful kidney transplants (done in the mid 1950s) were between identical twins, and both donors and recipients went on to lead normal lives.
- Use an "immunologically privileged" site. These are parts of the body where the immune system is prevented from mounting an attack. They include the eye, testes, and brain, but only the eye's privileged status has so far been exploited (for corneal grafts).
- Use a relative, preferably a sibling, as the donor. While never identical, they may have inherited some of the same histocompatibility antigens so the recipient's immune response may not be as strong as it otherwise would be.
- **Tissue-typing**. Determine the histocompatibility antigens of both recipient and potential donor and use the organ with the fewest mismatches.
- **Immunosuppression**. Use immunosuppressive agents to blunt the recipient's immune response. Invariably required for all **allografts**.

Tissue Typing

The strongest antigens expressed by tissues are the **class I** and **class II** histocompatibility molecules. These are encoded by an array of genes on **chromosome 6** called the **major histocompatibility complex** (**MHC**).

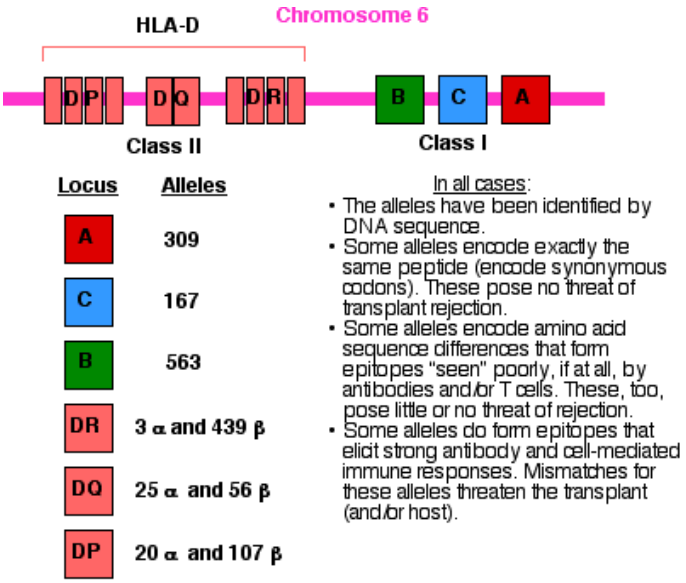


Figure 15.4.11.1 HLA

Class I molecules consist of a transmembrane protein to which are attached (noncovalently), a molecule of beta-2 microglobulin, and a short peptide. The class I transmembrane proteins are encoded by three loci: **HLA-A**, **HLA-B**, and **HLA-C**. Class I molecules are expressed at the surface of almost all the cells of the body (except for red blood cells and the cells of the central nervous system).

Class II molecules consist of two transmembrane polypeptides: an **alpha (α) chain and beta (β) chain** between which is nestled (noncovalently) a short peptide. The alpha and beta chains are encoded by clusters of loci in the region of chromosome 6 designated **HLA-D**. Unlike class I molecules, class II molecules are expressed on only a few types of cells, chiefly antigen-presenting cells (APCs) such as dendritic cells and macrophages, as well as other cells where inflammation is occurring.

Why so many MHC alleles

The genes of the MHC are the most polymorphic known. The graphic above shows the latest counts of alleles found at each locus in the human population. Of course, any one human can inherit a maximum of two alleles at each locus. The diversity of alleles in

the population makes possible thousands of different combinations. In a study of 1000 blood and organ donors in San Francisco that were typed for HLA-A and HLA-B,

- Over half the group had a combination that was unique.
- Another 111 donors had a set of these molecules that they shared with only one other person in the group.
- The most frequent phenotype (HLA-A1, HLA-A3, HLA-B7, and HLA-B8) was found in 11 donors.

The extreme polymorphism of the MHC did not evolve to frustrate transplant surgeons and their patients.

Why, then?

The function of class I and class II molecules is to "present" antigenic peptides to the T cells of the immune system. The peptides — usually about 9 amino acids long — are bound by noncovalent forces in a groove at the surface of the MHC molecule. These peptides can include fragments of protein antigens derived from intracellular pathogens (e.g. viruses). Different pathogens generate different antigenic fragments. Different MHC molecules differ in the efficiency with which they bind particular sequences of amino acids in these fragments. Therefore, we might expect that some MHC products would be better than others at presenting pathogen antigens to the immune system.

One piece of evidence: People infected with the human immunodeficiency virus (HIV-1) who have one particular HLA-B molecule are more resistant to developing a full-blown case of AIDS than those with other HLA-B molecules (even though some of these differ by only a single amino acid).

Another piece of evidence: Wegner and colleagues reported in the 5 September 2003 issue of *Science* the results of a direct test of this hypothesis. They exposed groups of sticklebacks — differing in the number of their class II alleles — to three types of parasite (all at once). They used two species of parasitic nematode and one species of trematode). Those fish with 5–6 different alleles resisted infection better than those with fewer (or more).

So it may well be that the great diversity of class I and class II alleles in the human population has helped ensure that no single pathogen can wipe out the entire population.

Techniques of tissue typing

Most tissue typing is done using serological methods: **antibodies** specific for those HLA antigens that have been identified in the human population. A reaction between cells of the subject and, for example, anti-HLA-A28 antibodies and HLA-A9 antibodies — but no other antibodies — establishes the phenotype. At the present time, routine typing is limited to establishing the phenotype at HLA-A, HLA-B, and HLA-DR.

Coming into wider use is **DNA typing**, especially for HLA-D antigens. It promises to improve the sensitivity and specificity of tissue typing. The totals of numbers of alleles at each HLA locus given in the graphic above are based on DNA typing.

How useful is tissue typing?

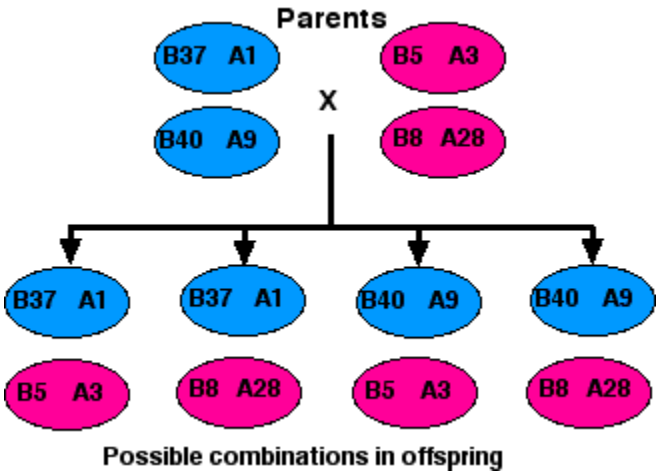


Figure 15.4.11.2 Possible combinations

So what hope do these data hold for the dialysis patient awaiting a kidney transplant? If the patient has a large family of willing donors, the odds for a good match are not bad because of the tight linkage of the HLA loci.

Assuming that no crossing over occurs within the HLA region of either the mother's or the father's two number 6 chromosomes, there are four possible combinations in which they may transmit their alleles to their children. So even if the parents carry different alleles at each locus (which is often the case), there is a 1:4 probability that any one of their children will be an exact match with any other. (Only the HLA-A and HLA-B antigens are shown here, but the tight linkage of the entire HLA region makes it likely that all the loci on each chromosome will be passed on as a block.)

But most organs are transplanted from cadavers to complete strangers. In the United States, the program is monitored by the **United Network for Organ Sharing** (**UNOS**). Tissue typing is usually limited to looking for **6** HLA antigens: the two each at **HLA-A**, **HLA-B**, and **HLA-DR**. If only one antigen is found at a locus, it means that either the tissue is homozygous for that allele or no reagent exists yet to detect the second allele. This table shows the results of one study of several thousand kidney recipients.

Number of HLA mismatches	**% kidneys surviving after 5 years**
0	68
1	61
2	61
3	58
4	58
5	57
6	56

The results tell us that:

- Having no mismatches provides a clear, but modest, advantage over mismatched kidneys. (This advantage is cumulative: at 17 years, 50% of the kidneys with no mismatches are still functioning while 50% of those with one or more mismatches have been lost after 8 years.)
- However, the incremental disadvantage of additional mismatches is small. In fact, the procedures to prevent rejection are now sufficiently good that ~90% of all kidneys — even those with all loci mismatched — can be expected to be functioning at the end of the first year.

Minor histocompatibility antigens

Even if it were possible to match donor and recipient at every locus of the MHC, some tissue incompatibility would still remain (except between identical twins). Few of the antigens responsible have been identified, but they include:

- **H-Y**, an antigen encoded on the Y chromosome and thus present in male, but not female, tissue
- **HA-2**, an antigen derived from the contractile protein myosin.

The number and variety of histocompatibility antigens tell us that probably no two humans (again, except for identical twins) exist on earth with perfectly compatible tissues. Therefore successful transplantation of allografts requires some degree of immunosuppression to avoid graft rejection.

Graft-versus-host disease (GVHD)

Allografts that contain **T cells** of the **donor** can cause graft-versus-host disease (**GVHD**). The T cells in the transplant see the host as "foreign" and proceed to mount a widespread attack against the tissues of the host. GVHD is an especially serious problem with grafts of **bone marrow** (the source of all blood cells) and cord blood. Even when the donor and recipient have identical HLA alleles, grafts of bone marrow often cause GVHD because of differences in their **minor histocompatibility antigens**.

Some cancer patients are now deliberately treated so vigorously with radiation and chemotherapy that their bone marrow is destroyed along with their cancer cells. In order to survive, these patients must be given stem cells to repopulate their marrow

after their therapy. In some cases, their own **bone marrow** is used. Some of it is removed prior to the onset of treatment of the patient and is itself treated to remove any cancer cells that may be lurking in it. If **allografted** bone marrow is required, there is a strong danger of GVHD. If the GVHD can be controlled, the stem cells should eventually establish themselves in the bone marrow of their new host and in due course generate some or even all of the patient's blood cells. **Cord blood** — another source of stem cells — presents less of a risk of serious GVHD even from a donor with HLA molecules not present in the recipient. This is because cord blood does not contain any mature T cells.

In mice, GVHD can be minimized by injecting large numbers of regulatory T cells, but for humans, control of GVHD — like control of graft rejection — still depends on the use of immunosuppression.

Immunosuppression

Immunosuppression is the treatment of the patient with agents that inhibit the immune response. The following is the list of immunosuppressants currently used.

Purine analogs

These are relatives of the purines used in DNA synthesis. Because they interfere with DNA synthesis, they interfere with the rapid cell proliferation needed for immune responses. **Azathioprine** (trade name = **Imuran**) is a widely-used purine analog.

Unfortunately, these drugs also interfere with the many other tissues that depend on rapid cell division (e.g., lining of the intestine, hair follicles) so they have many unpleasant side effects. Therefore, the search for agents that specifically target immune cells goes on.

Corticosteroids

These relatives of cortisol interfere with a transcription factor needed to turn on the genes for T cells to become activated. **Prednisone** and **prednisolone** are most commonly used.

Tacrolimus (Prograf®) and cyclosporine (Neoral®)

These are natural products isolated from microbial cultures. They inhibit the signaling pathway used by T cells to turn on their genes for activation, e.g., for IL-2 secretion.

Rapamycin

This is a macrolide antibiotic produced by an actinomycete found on Easter Island (which the inhabitants call Rapa Nui — hence the name). Rapamycin inhibits T cell proliferation, and shows great promise in reducing the problems of transplant rejection.

Rapamycin is also known as **sirolimus** and is sold under the trade name **Rapamune**.

Mycophenolate mofetil

This small molecule inhibits an enzyme needed by B and T cells for purine synthesis. Other types of cells are not dependent on the enzyme so side effects are mild. The trade name is **CellCept**.

Antithymocyte globulin (ATG)

This preparation contain antibodies — raised in horses or rabbits — directed against T cells.

Monoclonal antibodies

Several preparations are now used:

- **Muromonab-CD3 (OKT3)** and two humanized anti-CD3 monoclonals. They bind to the CD3 molecule on the surface of T cells.
- **Daclizumab** and **basiliximab**. Target the **IL-2 receptor** and thus inhibit only **activated T cells**.
- **Alemtuzumab** (Campath-1H®). Binds to CD52, a molecule found on lymphocytes and depletes both T cells and B cells.

Belatacept

This is a protein, produced by recombinant DNA technology, that combines

- the extracellular portion of CTLA-4 ("cytotoxic T-lymphocyte-associated antigen 4", one of the ligands for B7) with
- the Fc region (the C-terminal two-thirds of the constant region) of a human IgG1 antibody.

It blocks the "Signal Two" needed to activate T cells.

Side effects of immunosuppression

They are serious.

Infections

The immune system is vital to protect us against infectious agents (bacteria, viruses, fungi). So infection is a frequent side effect of immunosuppression in transplant recipients. Fortunately, the infections can usually be controlled by the appropriate antibiotic, antiviral drug, etc.

Cancer

5% or more of transplant recipients will develop cancer within a few years of receiving their allograft. This may not seem to represent a great risk, but it does represent a 100-fold increase in risk compared to the general population. Allograft recipients are particularly prone to developing skin cancers and lymphomas. Curiously, transplant recipients do not seem to be at any greater risk for developing the most common types of cancer in the rest of the population: cancers of the lung, breast, colon, and prostate. One exception: recipients of allografted **bone marrow** run a slightly, but significantly, higher (2–3 fold) risk of developing these types of tumors. In most cases, these cancers arise from a cell of the host. But in some cases of melanoma and Kaposi's sarcoma the cancer cells were present in the graft and proliferated in the immunosuppressed host.

Things that can be done to help in such cases include stopping the immunosuppression. The chief culprit seems to be the immunosuppression that these patients have been receiving. In most cases,this leads to regression of the cancer, but often rejection of the transplant as well. The choice is usually clear for patients with allografted kidneys; they can go back on dialysis and anticipate receiving another kidney at a future date. But what of the cancer patient with a heart transplant?

Future prospects for transplantation

Although organ transplants have helped thousands of people, much remains to be done. In particular, ways need to be found to

- increase the number of available organs (the need now far exceeds the supply)
- find more precise methods of immunosuppression in order to prevent rejection without the dangerous side effects of infection and cancer.

Both these problems may be helped by **xenotransplantation**.

Xenotransplantation

Xenotransplantation is the use of organs from other animals. A number of attempts have been made to use hearts, livers, and kidneys from such primates as chimpanzees and baboons — so far with limited success. One reason is that xenotransplants usually are attacked immediately by antibodies of the host resulting in **hyperacute rejection**. But perhaps the use of pigs as organ donors will be feasible.

- Their organs are about the right size for use in humans.
- They can be made transgenic for molecules that may circumvent
  - **hyperacute rejection** (by knocking out the genes responsible for cell-surface antigens that humans have preformed antibodies against);
  - the chronic, T-cell-mediated, rejection that plagues all allografts.
- They can be produced in the numbers needed.

However, pigs contain retroviruses (called **PERV** = porcine endogenous retrovirus), and there is fear that these might infect the human recipient.

Only a few transplants of pig tissue into humans have been done to date: skin grafts and grafts of pancreatic islets. A larger number of people have been temporarily hooked up to pig organs or "bioreactors" containing pig cells to provide support for their failing spleen, liver, or kidneys. Most of these recipients have been monitored for signs of infection by PERV and — even though PERV can infect human cells growing in culture — there is no evidence that any of these people exposed to pig tissue have become infected.

Is xenotransplantation safe?

Pigs are not the only animals that contain latent viruses in their cells. Could the viruses in the cells of other kinds of animal donors infect the transplant recipient? start an epidemic? The danger seems greater for xenotransplants from other **primates**. (**HIV**, the retrovirus that causes **AIDS** appears to have entered humans from a primate host)

For these reasons, many biologists are urging that transplant surgeons proceed cautiously with xenotransplants.

Immune Privilege

It has long been know that certain sites in the body, for example,

- the anterior chamber of the eye
- the testes
- the brain

are "privileged". They are protected from attack by the immune system.

This can cause problems. Several cases have emerged of survivors of Ebola who no longer have Ebola virus in their blood and are symptom-free but still retain live virus in such privileged sites as brain, testis, and aqueous humor of the eye where the virus has escaped attack by the immune system.

Many factors are involved in immune privilege, such as

- tight junctions between the cells of the tissue
- little expression of class I histocompatibility molecules
- expression of the Fas ligand, **FasL**.

The presence of FasL on their surface protects cells in privileged locations from immune attack because when threatened by a **cytotoxic T cell**, they force the T cell to commit suicide by apoptosis. Activated cytotoxic T cells express Fas on their surface. When they engage (with their T cell receptor) a privileged cell expressing FasL, instead of killing the target, the target kills them!

So if the organs of transgenic pigs can be made to expresses human FasL, perhaps they will be resistant to T-cell mediated rejection.

The human placenta also enjoys immune privilege. It is almost as foreign to the mother as a kidney transplant from her husband would be, but unlike the kidney, she will not reject it (at least not for 9 months). In lab rats, the embryos (and the mother's endometrium) secrete corticotropin-releasing hormone (CRH). This hormone induces the expression of Fas ligand (**FasL**) on the cells of the placenta. Activated T cells express **Fas**, so any threatening T cells would commit suicide by apoptosis when they encounter FasL on their target.

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15.4L: Bone Marrow Transplants

Transplants of Hematopoietic Stem Cells

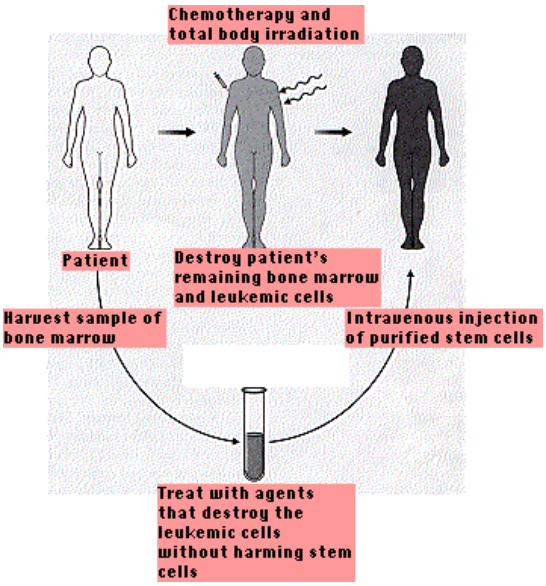


Figure 15.4.12.1 Bone marrow transplant

These can be:

- autologous — from hematopoietic stem cells that were
  - removed from the patient before cancer therapy began,
  - stored alive,
  - and, if there were cancer cells in the bone marrow (the case with multiple myeloma and leukemias), treated to "purge" them. Most failures of autologous stem cell transplants occur because of failure to get all the cancer cells out of the harvested cells rather than failure to eliminate them from the patient.
- **allogeneic** — hematopoietic stem cells removed from someone else, often a close relative. Another source of hematopoietic stem cells is **cord blood** — blood drained (through the umbilical cord) from the placenta of newborn infants.

  Allogeneic stem cells

  - avoid the problem of lurking residual cancer cells but
  - should be closely matched to the major histocompatibility loci (MHC) of the patient. If not, the donor cells will attack the recipient causing often-fatal **graft-versus-host disease** (GVHD). Even with an exact match at the MHC, some GVHD is likely.

In one remarkable case, an AIDS patient with leukemia was given a bone marrow transplant from a donor whose cells did not express a functional version of CCR5 — a coreceptor needed by HIV to infect T cells. Two years later, the patient was not only cured of his leukemia but of AIDS as well. Autologous hematopoietic stem cell transplants also show promise of being an effective treatment for the autoimmune disorder systemic lupus erythematosus (SLE). If the patient's own marrow was not completely destroyed, the donor lymphocytes and the patient's lymphocytes can exist together. Then a later infusion of the donor's T cells may be able to kill off all the patient's remaining malignant cells leaving the patient with a bone marrow that produces donor-type cells exclusively. So hematopoietic stem cell transplants (HSCT) can be life-saving but create their own problems. (Another example: an "immediate"-type allergy like hay fever or asthma of the donor can create the same allergy in the recipient.)

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15.4M: Antigen Presentation

Antigens are macromolecules that elicit an immune response in the body. Antigens can be proteins, polysaccharides, conjugates of lipids with proteins (lipoproteins) and polysaccharides (glycolipids). Most of this page will describe how **protein** antigens are presented to the immune system. The presentation of lipid and polysaccharide antigens will be mentioned at the end. It will be helpful to distinguish between two limiting cases.

Antigens that enter the body from the environment; these would include inhaled macromolecules (e.g., proteins on cat hairs that can trigger an attack of asthma in susceptible people), ingested macromolecules (e.g., shellfish proteins that trigger an allergic response in susceptible people), and molecules that are introduced beneath the skin (e.g., on a splinter or in an injected vaccine). Alternatively, antigens can be generated within the cells of the body; these would include proteins encoded by the genes of viruses that have infected a cell and aberrant proteins that are encoded by mutant genes; such as mutated genes in cancer cells. In all cases, however, the **initial immune response** to any antigen absolutely requires that the antigen be recognized by a T lymphocyte ("T cell"). The truth of this rule is clearly demonstrated in **AIDS**: the infections (viral or fungal or bacterial) that so often claim the life of AIDS patients do so when the patient has lost virtually all of his or her $CD4^+$ T cells. The two categories of antigens are processed and presented to T cells by quite different mechanisms.

Exogenous Antigens

Exogenous antigens (inhaled, ingested, or injected) are taken up by antigen-presenting cells (APCs). These include phagocytic cells like dendritic cells and macrophages and B lymphocytes ("B cells") which are responsible for producing antibodies against the antigen. Antigen-presenting cells

- engulf the antigen by endocytosis
- endosome fuses with a lysosome where the antigen is degraded into fragments (e.g. short peptides)
- these antigenic peptides are then displayed at the surface of the cell nestled within a **class II histocompatibility molecule**.
- they may be recognized by **$CD4^+$** T cells

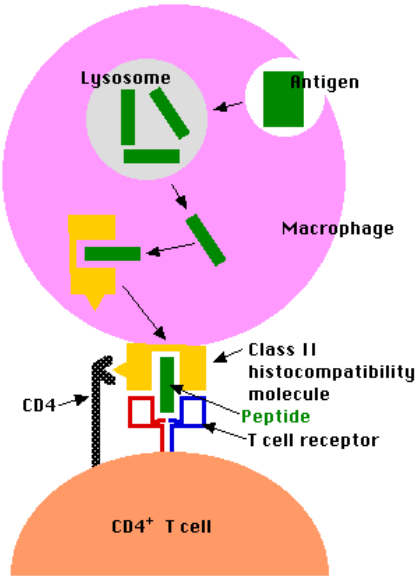


Figure 15.4.13.2 Class I histocompatibility molecule

The Class I Pathway

Class I histocompatibility molecules are transmembrane proteins expressed at the cell surface. Like all transmembrane proteins, they are synthesized by ribosomes on the rough endoplasmic reticulum (RER) and assembled within its lumen. There are three

subunits in each class I histocompatibility molecule:

- the transmembrane polypeptide (called the "heavy chain")
- the antigenic peptide
- beta-2 microglobulin

All of these must be present within the lumen of the endoplasmic reticulum if they are to assemble correctly and move through the Golgi apparatus to the cell surface. The Problem: proteins encoded by the genes of an infecting virus are synthesized in the **cytosol**. How to get them into the endoplasmic reticulum?

The Solution: **TAP** (= **t**ransporter **a**ssociated with antigen **p**rocessing).

- Viral proteins in the cytosol are degraded by **proteasomes** into viral peptides.
- The peptides are picked up by TAP proteins embedded in the membrane of the endoplasmic reticulum.
- Using the energy of ATP, the peptides are pumped into the lumen of the endoplasmic reticulum where they assemble with the transmembrane polypeptide and beta-2 microglobulin.
- This trimolecular complex then moves through the Golgi apparatus and is inserted in the plasma membrane.
- The complex can be bound by a **T cell** with a receptor (**TCR**) able to bind the peptide and flanking portions of the histocompatibility molecule (the hot dog in the bun) and **CD8** molecules that bind the **CD8 receptor** (shown above as a gray hemisphere) on the histocompatibility molecule.

The Class II Pathway

Class II histocompatibility molecules consist of two transmembrane polypeptides and a third molecule nestled in the groove they form. All three components of this complex must be present in the **endoplasmic reticulum** for proper assembly. But antigenic peptides are not transported to the endoplasmic reticulum, so a protein called the **invariant chain** ("**Ii**") temporarily occupies the groove.

The steps:

- The two chains of the class II molecule are inserted into the membrane of the endoplasmic reticulum.
- They bind (in their groove) one molecule of invariant chain.
- This trimolecular complex is transported through the Golgi apparatus and into vesicles called lysosomes.

Meanwhile foreign antigenic material is engulfed by endocytosis forming **endosomes**. These also fuse with lysosomes. Then,

- The antigen is digested into fragments.
- The invariant (Ii) chain is digested.
- This frees the groove for occupancy by the antigenic fragment.
- The vesicles move to the plasma membrane and the complex is displayed at the cell surface.
- The complex can be bound by a **T cell** with
  - a receptor (**TCR**) able to bind the peptide and flanking portions of the histocompatibility molecule (the hot dog in the bun) and
  - **CD4** molecules that bind the **CD4 receptor** (shown above as a yellow triangle) found on all class II histocompatibility molecules.

Interconnections Between the Class I and Class II Pathways

Cross-Presentation: Transferring Exogenous Antigens to the Class I Pathway

Cross-presentation is the transferring of extracellular antigens like bacteria, some tumor antigens, and antigens in cells infected by viruses into the class I pathway for stimulation of $CD8^+$ cytotoxic T cells (CTL). Only certain "professional" antigen-presenting cells (APCs) like dendritic cells can do this - use the class I as well as the class II pathways of antigen presentation.

Cross-presentation following infection by viruses is important because:

- Most viruses infect cells other than APCs (e.g., liver cells, epithelial cells of the lung) (and, of course, are intracellular in these).
- While viral antigens displayed on the surface of any infected cell can serve as **targets** for cytotoxic T cells (CTLs),
- the lack of any costimulatory molecules on the cell surface makes them **poor stimulants** for the development of clones of CTLs in the first place.

However, when an infected cell dies, it can be **engulfed** by a professional APC, and the antigens within it can enter the class I pathway. One mechanism:

- The dead cell is engulfed by endocytosis.
- The endosome that forms fuses with a lysosome and degradation of the dead cell begins.
- Antigens pass into the cytosol and are degraded in proteasomes.
- The peptides formed are then are picked up by TAP and inserted into class I MHC molecules and displayed at the cell surface — along with the costimulatory molecules needed to start a vigorous clonal expansion of $CD8^+$ cytotoxic T cells.

Diverting Antigens from the Class I to the Class II Pathway

Autophagy provides a mechanism by which cells can transfer **endogenous** (intracellular) antigens into the class II pathway, for example

- self-proteins so as to be able to delete $CD4^+$ T cells with receptors capable of attacking them and thus potentially capable of causing autoimmunity
- proteins synthesized by an infecting virus. In this way viral infection can generate **$CD4^+$ T cells** as well as cytotoxic T cells ($CD8^+$)

B Lymphocytes: A Special Case

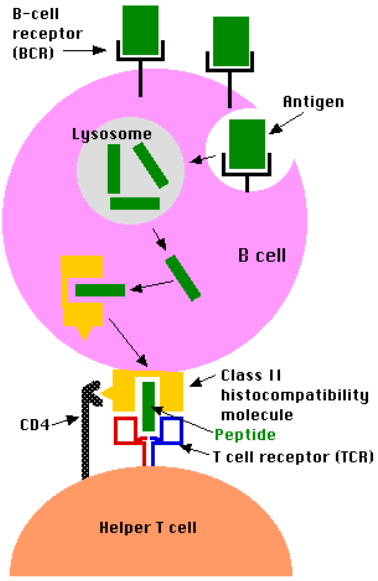


Figure 15.4.13.3 B-cell

B lymphocytes are both antigen-receiving and antigen-presenting cells. They bind intact antigens (e.g., virus particles, proteins) with their B cell receptor (BCR). They can come in contact with these antigens by encountering them in the surrounding lymph or by being presented them by macrophages or dendritic cells. B lymphocytes process antigen by the class II pathway for presentation to T cells.

The process:

- B cells engulf antigen by **receptor-mediated endocytosis**
- The B cell receptors for antigen (**BCR**s) are antibodies anchored in the plasma membrane.

- The affinity of these for an epitope on an antigen may be so high that the B cell can bind and internalize the antigen when it is present in body fluids in concentrations thousands of times smaller than a macrophage would need.
- The remaining steps of antigen processing occur by the same class II pathway described above for macrophages producing
- fragments of antigen displayed at the cell surface nestled in the groove of class II histocompatibility molecules.
- A $CD4^+$ T cell that recognizes the displayed antigen is stimulated to release lymphokines.
- These, in turn, stimulate the B cell to enter the cell cycle.
- Because of the part they play in stimulating B cells, these $CD4^+$ T cells are called **Helper T cells** ("**Th**").
- The B cell grows into a clone of cells (called **plasma cells**)
- These synthesize receptors (**BCR**s) with the **identical binding site** for the epitope but without the transmembrane tail.
- These **antibodies** are secreted into the surroundings.

Lipid and Polysaccharide Antigens

Lipid Antigens

- Lipid antigens are presented to T cells by cell-surface molecules designated **CD1** ("cluster of differentiation" 1).
- Antigen-presenting cells express several different forms of CD1 at their surface. Each is probably specialized to bind a particular type of lipid antigen (e.g. lipopeptide vs glycolipid).
- The exposed surface of CD1 molecules forms an antigen-binding groove much like that of MHC molecules except that
- the amino acids in the groove are more hydrophobic than those in MHC molecules.
- Like protein antigens, lipid antigens are also presented as fragments, i.e., as a "hot dog in a bun".

Polysaccharide Antigens

Some bacterial polysaccharides ingested by APCs

- can be degraded in their lysosomes
- and presented to T cells by MHC class II molecules.

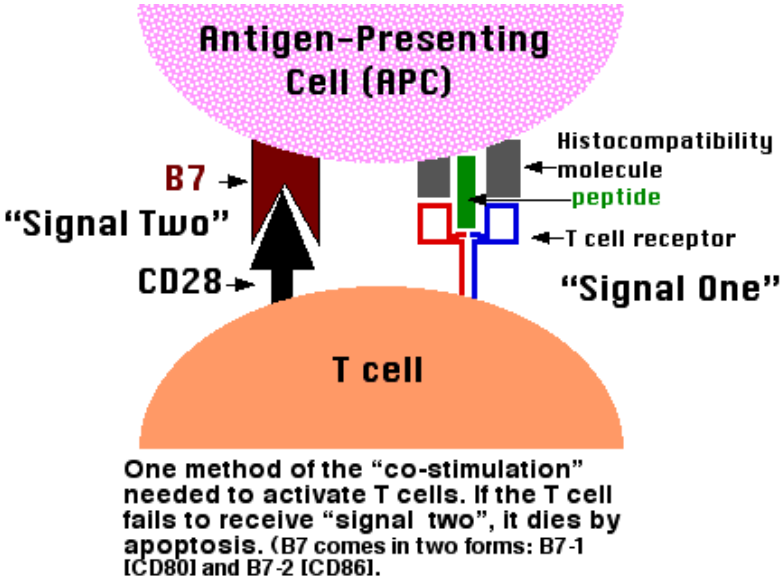


Figure 15.4.13.4 Co-stimulation

The binding of a T cell to an antigen-presenting cell (APC) is **by itself** not enough to activate the T cell and turn it into an effector cell: one able to, for examples,

- kill the APC ($CD8^+$ cytotoxic T lymphocytes [CTLs])
- carry out cell-mediated immune reactions ($CD4^+$ Th1 cells)

- provide help to B cells ($CD4^+$ Th2 cells)

In order to become activated, the T cell must not only bind to the epitope (MHC-peptide) with its TCR but also receive a **second signal** from the APC. The receipt of this second signal is called **costimulation**. Among the most important of these costimulators are molecules on the APC designated **B7** and their ligand on the T cell designated **CD28**. The binding of CD28 to B7 provides the second signal needed to activate the T cell.

Although T cells may encounter self antigens in body tissues, they will not respond unless they receive a second signal. In fact, binding of their TCR ("signal one") without "signal two" causes them to self-destruct by apoptosis. Most of the time, the cells presenting the body's own antigens either

- fail to provide signal two or
- transmit an as-yet-unidentified second signal that turns the T cell into a regulatory T cell (**Treg**) that suppresses immune responses.

In either case, self-tolerance results.

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15.4N: The Immunological Synapse

This page discusses the interactions in immunological responses, focusing on T cells and antigen-presenting cells (APCs). It highlights the roles of helper T cells and cytotoxic T lymphocytes, T-cell receptor (TCR) binding to MHC molecules, and the importance of costimulatory molecules, adhesion molecules, and cytokine receptors. These interactions are crucial for T cell activation and subsequent gene expression, resulting in the secretion of signaling molecules and cytotoxic factors.

Immunological responses, both antibody-mediated and cell-mediated involve close contact between a **T cell** and an **antigen-presenting cell** (**APC**).

Examples:

- Helper T cells (**Th1**) with "professional" **antigen-presenting cells** like **dendritic cells** and some **macrophages**
- Helper T cells (**Th2**) with **B cells**
- **Cytotoxic T lymphocytes** (**CTLs**) with their targets
- **NK cells** with their targets

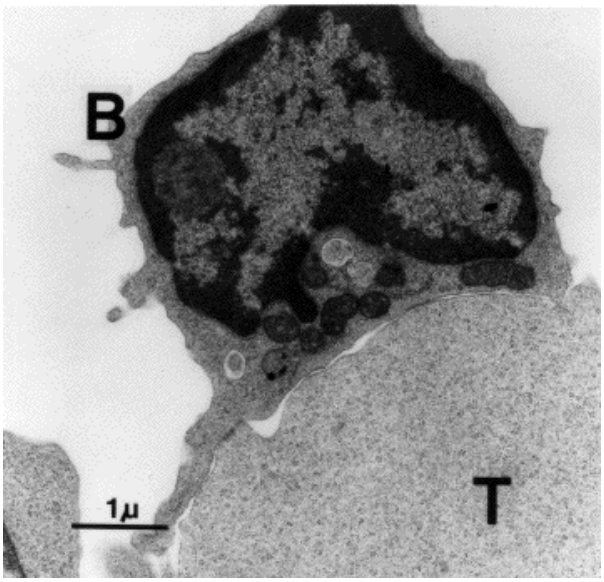


Figure 15.4.14.2 Pairs of molecules in immunosynapse

TCR/MHC-peptide pair

- On the **T cell**, the T-cell receptor for antigen (**TCR**) bound to
- a major histocompatibility complex (MHC) molecule on the **antigen-presenting cell** (APC).
    - For $CD4^+$ T cells, the MHC molecules are **class II**, and the binding is aided by CD4.
    - For $CD8^+$ T cells (e.g., CTLs), the MHC molecules are **class I**, and the binding is aided by CD8.
    - In both cases, the MHC molecule has an antigenic peptide nestled in an exterior groove (**MHC-peptide**).
- The TCR molecules are tethered by **actin filaments** in the cytoplasm.
- Several hundred TCR/MHC-peptide pairs are needed to stimulate a naive T cell to begin mitosis, but only 50 or so are needed to activate a "memory" T cell to do its work; that is, to become an effector T cell.

Costimulatory pairs

The costimulatory molecule **CD28** on the T cell bound to its ligand, **B7**, on the APC.

General adhesion molecules

- Leukocyte **F**unction-associated **A**ntigen-1 (**LFA-1**), an integrin on the T cell, bound to
- **I**nter**C**ellular **A**dehesion **M**olecule-1 (**ICAM-1**) on the APC.

Cytokine receptors

Cytokine receptors also cluster in the synapse (not shown in the diagram) where they are exposed to cytokines secreted into the synapse.

Formation of an immunological synapse causes the T cell to

- become activated with various signal pathways turning on new gene transcription
- release, by exocytosis, the contents of its vesicles:
  - Type 1 helper T cells (**Th1**): lymphokines like IFN-γ and TNF-β
  - Type 2 helper T cells (**Th2**): lymphokines like IL-4, IL-5, IL-10, and IL-13 that stimulate B cells
  - Cytotoxic T Lymphocytes (**CTLs**): cytotoxic molecules like **perforin** and **granzymes** that kill the target

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15.4O: Dendritic Cells

Dendritic cells (DCs) get their name from their surface projections (that resemble the dendrites of neurons). They are found in most tissues of the body and are particularly abundant in those that are interfaces between the external and internal environments (e.g., skin, lungs, and the lining of the gastrointestinal tract) where they are ideally placed to encounter extrinsic antigens, including those expressed by invading pathogens.

Although there are several distinct subtypes of DCs, they all share these features:

- They are actively motile.
- They continuously sample their surroundings — ingesting antigens by endocytosis (using phagocytosis, receptor-mediated endocytosis, and pinocytosis).
    - Many of these antigens are "self" antigens, e.g., dead cells, proteins in the extracellular fluid.
    - But the antigens can also be foreign antigens, for example, bacteria that are resident in the body (e.g., in the colon) or that invade the body.

    In either case, the ingested antigens are degraded in lysosomes into peptide fragments that are then displayed at the cell-surface nestled in class II MHC molecules.

Having ingested antigen in the tissue, they migrate to lymph nodes and spleen where they can meet up with T cells bearing the appropriate T-cell receptor for antigen (TCR).

What happens next depends on the nature of the antigen.

- Self antigens are presented to T cells without any costimulatory molecules. This interaction causes the T cells to divide for a brief time, but then they commit suicide by apoptosis and so cannot attack tissues of the body. The animal becomes tolerant to that antigen.
- Foreign antigens produce a different outcome. The dendritic cells becomes "activated' and begin to display not only
    - the MHC-peptide complex for the TCR of the T cells but also
    - costimulatory molecules, e.g. B7 which binds to CD28 on the T cell

The importance of dendritic cells in developing immunity to pathogens is dramatically shown in those rare infants who lack a functioning gene needed for the formation of dendritic cells. They are so severely immunodeficient that they are at risk of life-threatening infections.

What accounts for the activation of dendritic cells by foreign antigens but not by self antigens? Pathogens, especially bacteria, have molecular structures that

- are not shared with their host
- are shared by many related pathogens
- are relatively invariant; that is, do not evolve rapidly (in contrast, for example, to such pathogen molecules as the hemagglutinin and neuraminidase of influenza viruses).

These are called Pathogen-Associated Molecular Patterns (PAMPs)

Examples:

- the flagellin of bacterial flagella
- the peptidoglycan of Gram-positive bacteria
- the **lipopolysaccharide** (**LPS**, also called **endotoxin**) of Gram-negative bacteria
- double-stranded RNA. (Some viruses of both plants and animals have a genome of dsRNA. And many other viruses of both plants and animals have an RNA genome that in the host cell is briefly converted into dsRNA).
- unmethylated DNA (eukaryotes have many times more cytosines, in the dinucleotide CpG, with methyl groups attached).

Dendritic cells have a set of transmembrane receptors that recognize different types of PAMPs. These are called Toll-like receptors (TLRs) because of their homology to receptors first discovered and named in Drosophila.

TLRs identify the nature of the pathogen and turn on an effector response appropriate for dealing with it. These signaling cascades lead to the expression of various **cytokine genes**.

- **Interleukin 12** (IL-12) drives the nearby T cells to become Th1 cells, which will provide help for cell-mediated immunity including attack against **intra**cellular pathogens.
- **IL-23**, which promotes differentiation of the T cells into Th17 helper cells, which can deal with **extra**cellular bacteria.
- **IL-4**, which promotes differentiation of the T cells into Th2 cells which provide help for antibody production by B cells.

Under other circumstances, activated dendritic cells may secrete TGF-β and **IL-10**, leading to the formation of regulatory T cells (Treg) that dampen immune responses.

Dendritic Cell Subsets

While all DCs share certain features, they actually represent a variety of cell types with different differentiation histories, phenotypic traits and, as outlined above, different effector functions.

Examples:

Myeloid Dendritic Cells

As their name implies, these cells ("mDCs") are derived from the same myeloid progenitors in the bone marrow that give rise to granulocytes and monocytes. They present antigen to T cells and activate the T cells by secreting large amounts of IL-12.

Plasmacytoid Dendritic Cells

These cells ("pDCs") get their name from their extensive endoplasmic reticulum which resembles that of plasma cells. However, unlike plasma cells that are machines for pumping out antibodies, pDCs secrete huge amounts of interferon-alpha especially in response to viral infections.

Plasmacytoid DCs have internal toll-like receptors:

- TLR-7 and TLR-8, which bind to the single-stranded RNA (ssRNA) genomes of such viruses as influenza, measles, and mumps.
- TLR-9, which binds to the **un**methylated cytosines in the dinucleotide CpG in the DNA of the pathogen. (The cytosines in the host's CpG dinucleotides often have methyl groups attached.)

$CD8^+$ vs. $CD8^-$ Dendritic Cells

These subsets are found in the mouse spleen.

- The $CD8^-$ subset presents antigen engulfed from the surroundings — using the class II pathway — to $CD4^+$ helper T cells.
- The $CD8^+$ subset can present extracellular antigens using the class I pathway as well. The peptide/MHC class I molecules are presented to $CD8^+$ T cells which go on to become cytotoxic T lymphocytes (CTL). This phenomenon is called **cross-presentation**.

Dendritic cells can also present undegraded antigen to B cells; that is, antigen that has not been processed into peptide/MHC complexes.

Monocyte-derived Dendritic Cells (Mo-DCs)

Humans (and mice) have another population of dendritic cells that develop from blood-borne monocytes that have been exposed to Gram-negative bacteria (or their LPS). The LPS is detected by their TLR4 molecules. Mo-DCs can present antigen to **both** $CD4^+$ T cells and $CD8^+$ T cells (cross-presentation).

Ralph Steinman

Ralph Steinman, the pioneer in the study of dendritic cells, has provided striking visual evidence of the cellular interactions between antigen-presenting dendritic cells, T cells, and B cells. When spleen cells are cultured with antigen, tight clusters of cells form (see figure). The clustering occurs in two phases:

- an early phase (days 0–2) during which only the dendritic cells and T cells need to be present to form clusters
- a later phase (days 2–5) when antigen-primed B cells enter the cluster and differentiate into antibody-secreting cells

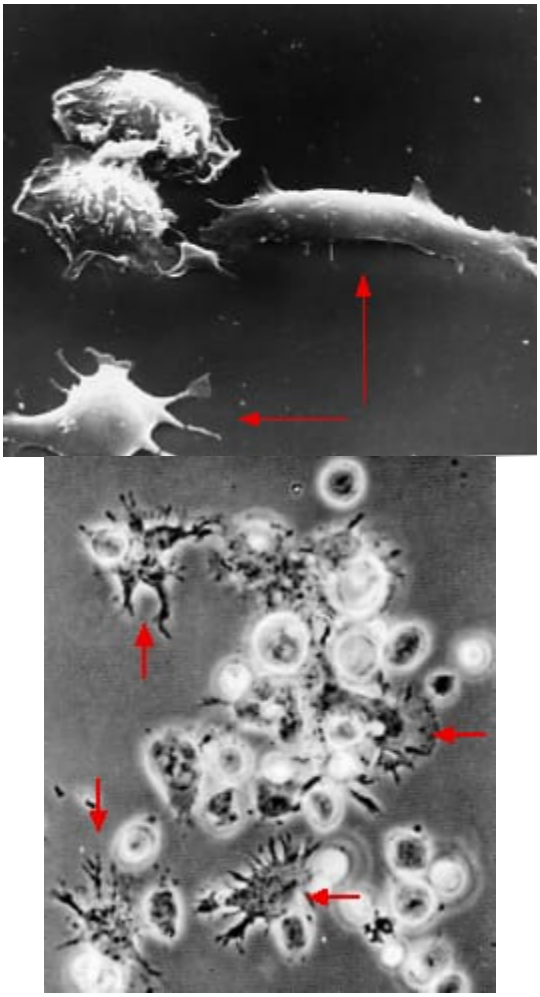


Figure 15.4.15.2 spleen cells after 2 days in culture. Four dendritic cells (arrows)

Pictures Courtesy of Ralph Steinman from K. Inaba *et al.*, **J. Exp. Med. 160**:858, 1984

Homing

Some dendritic cells not only activate T cells to respond to a particular antigen but tell them where to go to deal with that antigen.

Two examples:

Antigens in the skin

- Dendritic cells engulf antigens in (or even on!*) the skin and, while doing so, convert calciferol (vitamin D3) present in the skin into calcitriol (1,25[OH]2 Vitamin D3).

- When they activate the appropriate T cells in a nearby lymph node, the calcitriol induces those T cells to express a surface receptor designated CCR10 (a member of the CC chemokine receptor family).
- CCR10 binds the chemokine CCL27 — which is present in the skin.
- So when these T cells reajavascript:void('Remove Anchor')ch the skin, they stop their travels and go to work there.

Antigens in the GI tract

- Dendritic cells in the lining of the intestine are always busy engulfing the many antigens present there.
- While doing so, they convert the abundant amount of retinol (vitamin A) there into retinoic acid.
- When they activate the appropriate T cells in a nearby lymph node, the retinoic acid induces those T cells to express another CC chemokine receptor designated CCR9.
- CCR9 binds the chemokine CCL25 present in the intestine.
- So when these T cells reach the intestine, they stop their travels and go to work. (CCL25 also attracts IgA-secreting B cells.)

Switching Homing Directions

Most vaccines are given by injection into muscle or skin. This works very well for inducing systemic immunity; that is, IgG antibodies in the blood able to attack pathogens (e.g., tetanus) that are present in the blood.

Injected vaccines do not work as well for illnesses caused by intestinal pathogens such as

- typhoid fever (caused by *Salmonella typhi*)
- cholera (caused by *Vibrio cholerae*)

However, a group of German immunologists reported in July 2011 that:

- whereas dendritic cells receiving antigens from injections under the skin influence T cells to migrate back to the skin (as we saw above),
- if these subcutaneous injections are accompanied by injections of **retinoic acid**, the T cells migrate to the intestine instead.
- $CCR9^+$ plasma cells secreting antigen-specific IgA antibodies also appeared in the intestine.

Using this technique, these workers were able to protect their mice from mouse typhoid (*Salmonella typhimurium*) and cholera toxin.

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15.4P: Passive Immunity



Figure 15.4.16.1: Milking a snake for the production of antivenom. from Wikipedia (CC-BY-2.0; Barry Rogge).

Immune Globulin (IG)

Horse and sheep proteins are foreign to the human patient and will, in due course, elicit an active immune response. This may lead to an allergic reaction such as systemic anaphylaxis or serum sickness. To avoid such problems, humans are often used as the source of passive antibodies.

- IG is also used to provide protection to boys with **X-linked agammaglobulinemia**, who are unable to manufacture antibodies because of a mutation in their single (because on their X chromosome) gene for Bruton's tyrosine kinase.
- hepatitis A ("infectious" hepatitis), measles, and rubella. Some **immune globulin** (IG) is prepared from the gamma globulin fraction of pooled plasma from the outdated blood of several thousand blood donors on the assumption that this large pool will contain good levels of antibodies against many common diseases such as
- Some preparations of immune globulin are harvested from selected **individual** donors who have either recently recovered from the disease or who have been deliberately and intensively immunized against it. These are used to provide immediate protection against such diseases as rabies, tetanus, varicella (chicken pox), and complications arising from giving the smallpox vaccine (vaccinia immune globulin or VIG).
- The recent need for an effective treatment for people with **inhalational anthrax** has led to the use of **plasma** donated by military personnel previously actively immunized with anthrax vaccine. Soon it should be possible to prepare a purified immune globulin from this plasma. Further down the road will be the use of antianthrax monoclonal antibodies.
- Rh immune globulin (**RhIg**) or **Rhogam** is used to prevent Rh-negative mothers from becoming sensitized to the Rh antigen of their newborn child.

Advantages of human immune globulin

The preparation contains fewer irrelevant serum proteins and of those that remain, being human proteins, they are far less immunogenic and are catabolized more slowly than horse proteins. However, care must be (and is) taken to ensure that the preparations are not contaminated with human pathogens such as the AIDS virus (HIV) or hepatitis viruses.

Non-antigen-specific effects of human immune globulin

Intravenous injections of IG have helped patients with such autoimmune disorders as

- immune hemolytic anemia
- immune thrombocytopenic purpura
- myasthenia gravis

The therapeutic effect seems to have nothing to do with the antigen specificities (e.g., antitetanus) of the antibodies in the preparation. Instead it is the C-region portion of the antibody molecules that provides the protection. Animal studies suggest that it does so by binding to a class of receptors on macrophages, which inhibits them from phagocytosing antibody-coated cells, e.g.,

- antibody-coated red cells in immune hemolytic anemia
- antibody-coated platelets in idiopathic thrombocytopenic purpura

The spleen is packed with macrophages and is where most of red blood cell and platelet destruction occurs in these diseases (and explains why removal of the spleen so often helps the patient).

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15.4Q: Innate Immunity

The ability of a multicellular organism to defend itself against invasion by pathogens (bacteria, fungi, viruses, etc.) depends on its ability to mount immune responses. All metazoans (probably) have inborn defense mechanisms that constitute innate immunity. Vertebrates have not only innate immunity but also are able to mount defense mechanisms that constitute adaptive immunity. This table gives some of the distinguishing features of each type of immunity.

Innate Immunity	Adaptive Immunity
Pathogen recognized by receptors encoded in the germline	Pathogen recognized by receptors generated randomly
Receptors have broad specificity, i.e., recognize many related molecular structures called PAMPs (**p**athogen-**a**ssociated **m**olecular **p**atterns)	Receptors have very narrow specificity; i.e., recognize a particular epitope
PAMPs are essential **polysaccharides** and **polynucleotides** that differ little from one pathogen to another but are not found in the host.	Most epitopes are derived from **polypeptides** (proteins) and reflect the individuality of the pathogen.
Receptors are PRRs (**p**attern **r**ecognition **r**eceptors)	In jawed vertebrates, the receptors are B-cell (**BCR**) and T-cell (**TCR**) receptors for antigen
Immediate response	Slow (3–5 days) response (because of the need for clones of responding cells to develop)
Little or no memory of prior exposure	Memory of prior exposure
Occurs in all metazoans	Occurs in vertebrates only

The Cells of the Innate Immune System

A variety of different types of cells participate in innate immunity. What they all have in common is that the receptors by which they recognize pathogens are limited in their specificity. This is in contrast to the B cells and T cells of the adaptive immune system that generate receptors — BCRs and TCRs respectively — that are exquisitely specific for the pathogen. The players:

- The several granulocytes of the blood and tissues
  - neutrophils
  - eosinophils
  - basophils and mast cells
- monocytes and macrophages
- dendritic cells
- **Innate Lymphoid Cells** (ILCs). These are cells that look like lymphocytes but do not have the antigen receptors found on B lymphocytes (BCRs) and T lymphocytes (TCRs). They include cytotoxic Natural Killer (NK) cells and several subsets of non-cytotoxic cells (ILC1, ILC2, ILC3, etc.) each with it own pattern of cytokine secretion and favored targets.

Pathogen-Associated Molecular Patterns (PAMPs)

Pathogens, especially bacteria, have molecular structures that are not shared with their host and are shared by many related pathogens. They are relatively invariant; that is, do not evolve rapidly (in contrast, for example, to such pathogen molecules as the hemagglutinin and neuraminidase of influenza viruses).

Examples:

- the flagellin of bacterial flagella
- the peptidoglycan of Gram-positive bacteria
- the **lipopolysaccharide** (**LPS**, also called **endotoxin**) of Gram-negative bacteria
- double-stranded RNA. (Some viruses of both plants and animals have a genome of dsRNA. And many other viruses of both

plants and animals have an RNA genome that in the host cell is briefly converted into dsRNA).
- unmethylated DNA (eukaryotes have many times more cytosines, in the dinucleotide CpG, with methyl groups attached).

Pattern Recognition Receptors (PRRs)

There are three groups:

1. secreted molecules that circulate in blood and lymph;
2. surface receptors on phagocytic cells like macrophages that bind the pathogen for engulfment;
3. cell-surface receptors that bind the pathogen initiating a signal leading to the release of effector molecules (cytokines).

Secreted PRRs

Example: Circulating proteins (e.g., C-reactive protein) that bind to PAMPs on the surface of many pathogens. This interaction triggers the complement cascade leading to the opsonization of the pathogen and its speedy phagocytosis.

Phagocytosis Receptors

Macrophages have cell-surface receptors that recognize certain PAMPs, e.g., those containing mannose. When a pathogen covered with polysaccharide with mannose at its tips binds to these, it is engulfed into a phagosome.

Toll-Like Receptors (TLRs)

Macrophages, dendritic cells, and epithelial cells have a set of transmembrane receptors that recognize different types of PAMPs. These are called Toll-like receptors (TLRs) because of their homology to receptors first discovered and named in Drosophila. Mammals have 12 different TLRs each of which specializes — often with the aid of accessory molecules — in a subset of PAMPs. In this way, the TLRs identify the nature of the pathogen and turn on an effector response appropriate for dealing with it. These signaling cascades lead to the expression of various **cytokine genes**. Examples:

- **TLR-1:** Forms a heterodimer with TLR-2 at the cell surface which binds to the peptidoglycan of Gram-positive bacteria like Streptococci and Staphylococci.
- **TLR-2:** With TLR-1, binds cell-wall components of Gram-positive bacteria.
- **TLR-3:** Binds to the double-stranded RNA of viruses engulfed in endosomes.
- **TLR-4:** Activated by the lipopolysaccharide (endotoxin) in the outer membrane of Gram-negative bacteria like Salmonella and *E. coli* **O157:H7**
- **TLR-5:** Binds to the **flagellin** of motile bacteria like Listeria.
- **TLR-6:** Forms a heterodimer with TLR-2 and responds to peptidoglycan and certain lipoproteins.
- **TLR-7 and TLR-8:** Form a heterodimer that binds to the single-stranded RNA (ssRNA) genomes of such viruses as influenza, measles, and mumps that have been engulfed in endosomes.
- **TLR-9:** Binds to the **un**methylated CpG of the DNA of bacteria that have been engulfed in endosomes. (The cytosines in the host's CpG dinucleotides often have methyl groups attached.)
- **TLR-11:**In mice, it binds proteins expressed by several infectious protozoans (Apicomplexa) as well as, like TLR-5, to flagellin. Humans do not have TLR-11.

In all these cases, binding of the pathogen to the TLR initiates a signaling pathway leading to the activation of NF-κB. This transcription factor turns on many cytokine genes such as those for tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and chemokines, which attract white blood cells to the site. All of these effector molecules lead to **inflammation** at the site. And even before these late events occur, the binding of Gram-positive bacteria to TLR-2 and Gram-negative bacteria to TLR-4 enhances phagocytosis and the fusion of the phagosomes with lysosomes.

Innate Immunity can trigger Adaptive Immunity

This can occur in several ways:

Macrophages and **dendritic cells** are phagocytes and are also responsible for "presenting" antigens to T cells to initiate both

cell-mediated and antibody-mediated adaptive immune responses.

- Digested fragments of the engulfed pathogen are returned to the cell surface nestled in the cavity of class II histocompatibility molecules.
- Gene transcription turned on by the interaction of PAMPs and TLRs causes transmembrane molecules called **B7** to appear at the cell surface.
- T cells have a receptor for B7 called CD28.
- Simultaneous binding of
  - CD28 to B7 and
  - the antigen/class II complex to TCRs specific for it
- activates the T cell.
- This leads to repeated mitotic divisions producing **clones** of $CD4^+$ T cells that can carry out cell-mediated immune responses and/or stimulate B cells to secrete antibodies of the appropriate specificity

Dendritic cells also engulf self-antigens, e.g., body cells that have died by apoptosis, but because these have no PAMPs associated with them, there is no second signal to activate the T cells.

The interaction of PAMPs and TLRs on **dendritic cells** causes them to secrete cytokines, including

- **interleukin 12** (IL-12) which stimulates the production of Th1 cells
- **interleukin 23** (IL-23) which stimulates the production of Th17 cells
- **interleukin 6** (IL-6), which interferes with the ability of **regulatory** T cells to suppress the responses of **effector** T cells to antigen. A double-negative is a positive.

B cells are also antigen-presenting cells. They bind antigen with their BCRs and engulf it into lysosomes. They then transport the digested fragments to the cell surface incorporated in class II histocompatibility molecules just as macrophages and dendritic cells do. B cells also have TLRs. When a PAMP such as LPS binds the TLR, it enhances the response of the B cell to the antigen.

It has been known for many years that for vaccines to be effective, the preparation must contain not only the antigen but also materials called **adjuvants**. Several adjuvants contain PAMPs, and their stimulus to the innate immune system enhances the response of the adaptive immune system to the antigen in the vaccine. Pathogens coated with fragments of the complement protein C3 are not only opsonized for phagocytosis but also bind more strongly to B cells that have bound the pathogen through their BCR. This synergistic effect enables antibody production to occur at doses of antigen far lower than would otherwise be needed. Some workers feel that, in fact, adaptive immunity is not possible without the assistance of the mechanisms of innate immunity.

Antimicrobial Peptides

In addition to their innate pathogen-**recognition** systems, vertebrates (including ourselves), invertebrates (e.g., Drosophila), even plants and fungi secrete **antimicrobial peptides** that protect them from invasion by bacteria and other pathogens. In fact, probably all multicellular organisms benefit from this form of **innate immunity**. For humans, the best-studied antimicrobial peptides are the **defensins**, hepcidin and the **cathelicidins**

Defensins

All our epithelial surfaces

- skin
- lining of the GI tract
- lining of the genitourinary tracts
- lining of the nasal passages and lungs

are protected by defensins.

- Some defensins are secreted by the epithelial cells themselves; others by Th17 cells and neutrophils.
- Some are secreted all the time; others only in response to attack by pathogens. (In some cases their genes are turned on by activated TLRs.)

- They are synthesized from larger gene-encoded precursors which are
- cut to produce the active peptide.
- These range in length from 25 to 45 amino acids.
- In humans, they contain 6 invariant cysteines that form 3 disulfide bonds that assist in producing a secondary structure that consists of 3 strands of anti-parallel **beta sheet**.
- They attack the outer surface of the cell membrane surrounding the pathogen eventually punching lethal holes in it. (Unlike eukaryotes, the phospholipids in the outer membrane of bacteria carry a surplus of negative charges, and the positive charges on the defensins probably enable them to penetrate the bacterial membranes while sparing host membranes.)

Curiously, some defensins (β-defensin) also affect coat color (in dogs and mice) and in other ways mimic the effects of melanocyte-stimulating hormone (MSH).

Hepcidin

Hepcidin is a peptide of 25 amino acids with a secondary structure (beta sheet) like that of the defensins. It is secreted by the liver and controls the level of iron in the blood and ECF by regulating its release from intracellular stores. Hepcidin secretion is increased in response to invasion by pathogens (fungi and bacteria). Many of these require iron for their virulence and by blocking the release of iron into the blood, hepcidin starves them of this essential factor.

Cathelicidins

The best known human cathelicidin is LL37, a peptide of 37 amino acids synthesized by macrophages, neutrophils, adipocytes, and epithelial cells (providing antimicrobial protection to our skin and the lining of our urinary tract). Unlike the defensins, its secondary structure is **alpha helix**.

Like defensins, the gene for LL37 can be turned on by activated TLRs. In macrophages, for example, cathelicidin synthesis within the cell promotes killing of engulfed bacteria like M. tuberculosis, the agent of TB. Activation of the cathelicidin gene requires the presence of the active form of vitamin D (1,25 $[OH]_2$ vitamin D_3). This may explain:

- why people with a deficiency of vitamin D are more susceptible to tuberculosis;
- the physiological basis for the practice of exposing patients to sunlight in TB sanitariums (before the days of antibiotics).

Antimicrobial Peptides and the GI Tract

The contents of the GI tract (especially the colon) are loaded with bacteria. But most of these cause no trouble thanks to a variety of defenses. Among these is the barrier of antimicrobial peptides that exists from mouth to anus.

- The epithelium of the mouth and tongue is protected by a layer of antimicrobial peptides as well as those secreted in the saliva.
- The stomach is also protected by antimicrobial peptides (cut by **pepsin** from a larger precursor) as well as by the low pH of **gastric juice**.
- The liquefied contents that leave the stomach are quickly neutralized by the bicarbonate ions in the pancreatic fluid. However, any bacteria that survived the trip through the stomach (e.g., *E. coli* has a proton pump that enables it to survive the strong acid of the gastric juice) are kept in check by the antimicrobial peptides secreted by the **Paneth cells** of the small intestine. So, the contents of the small intestine normally contain only a small population of microbes.
- Not so for the large intestine (colon). The colon supports an enormous population ($>10^{13}$) of microorganisms, but these seldom invade its lining thanks to
  - a protective barrier of antimicrobial peptides as well as
  - the protective actions of continuous stimulation of
    - TLR-2s by Gram-positive commensals and
    - TLR-4s by Gram-negative commensals
- The rectum is also protected by an epithelial barrier of antimicrobial peptides.

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15.4R: The Complement System

Sometimes the interaction of antibodies with antigen is useful by itself. For example,

- coating a virus or bacterium thus preventing it from binding to — and invading — a host cell (e.g., antipolio antibodies)
- binding to a toxin molecule (e.g., diphtheria or tetanus toxin) thus keeping the toxin from entering a cell where it does its dirty work

But most of the time, the binding of antibodies to antigen performs no useful function until and unless it can activate an **effector mechanism**. The complement system serves several effector roles. The complement system provides the actual protection from the response while the interaction of antibodies and antigen provides the **specificity** of the response. Put another way, antibodies "finger" the target, complement destroys it.

Features of the system

- The complement system consists of some 30 proteins circulating in blood plasma.
- Most of these are inactive until they are cleaved by a protease which, in turn, converts them into a protease.
- Thus many components of the system serve as the substrate of a prior component and then as an enzyme to activate a subsequent component.
- This pattern of sequential activation produces an expanding cascade of activity (reminiscent of the operation of the blood clotting system).

The Classical Pathway

The binding of antibody to its antigen often triggers the complement system through the so-called classical pathway. It can occur in solution or — as shown here — when the antibodies have bound to antigens on a cell surface.

The proteins of the classical pathway

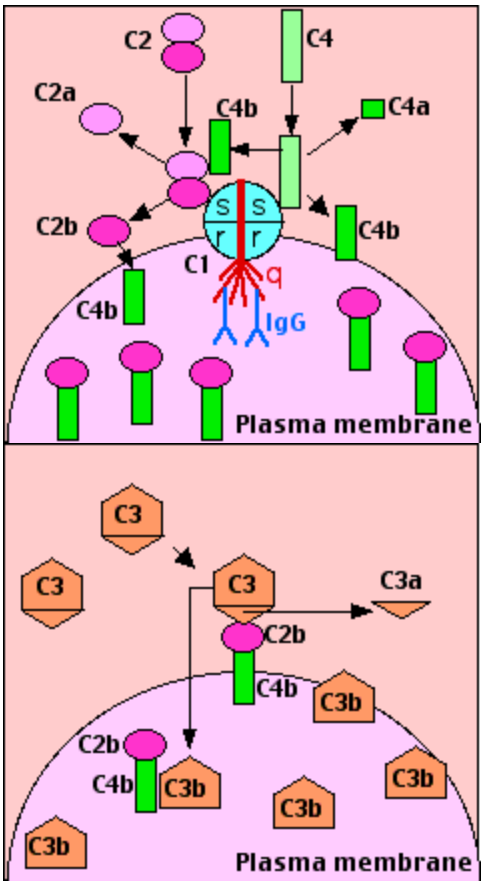


Figure 15.4.18.1 Classical pathway

C1

C1 exists in blood serum as a molecular complex containing:

- 6 molecules of C1q
- 2 molecules of C1r
- 2 molecules of C1s

The constant regions of mu chains (IgM) and some gamma chains (IgG) contain a binding site for C1q. A single molecule of IgM is enough to initiate the pathway. IgG is far less efficient, requiring several molecules to do so (6 is the optimum — the same as the number of C1q molecules in C1).

- Binding of C1q activates **C1s** and **C1r**.
- Activated C1s (a serine protease) cleaves two serum proteins:
  - **C4** is cleaved into a large fragment
    - **C4b**, which binds covalently to sugar residues on cell-surface glycoproteins, and a smaller, inactive, fragment of
    - C4a which diffuses away.
  - **C2** is cleaved into
    - **C2b**, which binds noncovalently to a site on **C4b**, leaving a smaller, inactive, fragment of
    - C2a which diffuses away.
  - The complex of **C4b•2b** is called "**C3 convertase**" because it catalyzes the cleavage of **C3**. (C4b•2b is also a serine protease.)

C3

C3 is the most abundant protein of the complement system (~1.3 mg/ml). Because of its abundance and its **ability to activate itself**, it greatly magnifies the response.

- **C4b•2b** cuts **C3** into two major fragments:
  - **C3b**, which binds covalently to glycoproteins scattered across the cell surface. Macrophages and neutrophils have receptors for **C3b** and can bind the C3b-coated cell or particle preparatory to phagocytosis. This effect qualifies C3b as an **opsonin**.
  - **C3a** This small fragment is released into the surrounding fluids. It can bind to receptors on basophils and mast cells triggering them to release their vasoactive contents (e.g., histamine). Because of the role of these materials in anaphylaxis, C3a is called an **anaphylatoxin**.
- Some of the C3b binds to molecules of C5 creating an allosteric change that exposes them to cleavage by C4b•2b (which is thus a "C3/C5 convertase".)

C5

Cleavage of C5 by the C3/C5 convertase initiates the assembly of a set of complement proteins that make up the membrane attack complex. (The membrane attack complex can also be formed by another C5 convertase produce by the "alternative pathway".)

The Membrane Attack Complex

Cleavage of C5 by the C3/C5 convertase, produces:

- **C5a**, which is released into the fluid surroundings where it
  - is a potent anaphylatoxin (like C3a)
  - is a chemotactic attractant for neutrophils
- **C5b**, which serves as the anchor for the assembly of a single molecule each of C6, C7 and C8.
- The resulting complex **C5b•6•7•8** guides the polymerization of as many as 18 molecules of **C9** into a tube inserted into the lipid bilayer of the plasma membrane. This tube forms a channel allowing the passage of ions and small molecules. Water enters the cell by osmosis and the cell lyses.

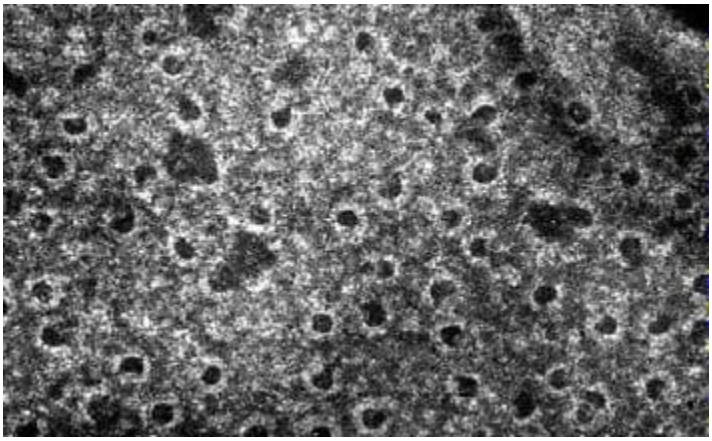


Figure 15.4.18.2 Holes punched in a membrane by complement system

The electron micrograph (courtesy of Drs. J. H. Humphrey and R. Dourmashkin) shows holes punched through the cell wall of the Gram-negative bacterium ***Shigella dysenteriae*** by the terminal components of the complement system. (Some of the holes are larger than expected for C9 channels and probably were enlarged later by the action of lysozyme.)

Effector Functions of Complement

Cell lysis is only one function (and probably not the most important one) of the complement system. The complement system acts in several ways to mobilize defense mechanisms.

- **Opsonization** by C3b targets foreign particles for phagocytosis.
- **Chemotaxis** by C5a attracts phagocytic cells to the site of damage.
- This is aided by the **increased permeability** of the capillary beds mediated by **C3a** and **C5a**.

- The early complement components are also important for **solubilizing** antigen-antibody complexes assisting in their catabolism and elimination from the body. Failure of this function can lead to immune complex disorders.
- **Lysis** of antibody-coated cells. (In some cases, this causes more harm than good; complement-mediated lysis can cause such serious disorders as
  - Rh disease
  - immune hemolytic anemia
  - immune thrombocytopenic purpura
- Promoting antibody formation. Breakdown of C3b generates a fragment (C3d) that binds to antigens enhancing their uptake by dendritic cells and B cells.

  (The C3d·antigen complex binds to the same receptor on B cells that the Epstein-Barr virus (EBV) uses to gain entry into B cells — where it may cause mononucleosis and, sometimes, Burkitt's lymphoma.)

The Alternative Pathway

The complement system can also be triggered without antigen-antibody complexes. Even in their absence, there is a **spontaneous** conversion of C3 to C3b. Ordinarily the C3b is quickly inactivated: the C3b binds to inhibitory proteins and **sialic acid** present on the surface of the body's own cells, and the process is aborted. However, bacteria and other foreign materials that may get into the body lack these proteins and have little or no sialic acid.

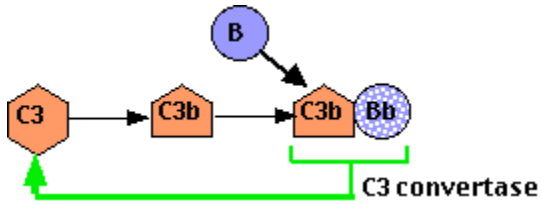


Figure 15.4.18.3 C3b

So the C3b

- binds a protein called Factor B forming a complex of **C3b•Bb**.
- C3b•Bb is also a **C3 convertase** acting on more C3 to form:
  - C3b•Bb•C3b, which is a **C5 convertase** and can start the assembly of the membrane attack complex.
  - more C3b! This second function (shown here) creates a positive feedback loop, amplifying what might have started as a small reaction (the formation of C3b by either or both the classical and alternative pathways) into a massive production of C3b.

Regulation of Complement Activity

The explosive potential of the complement system requires that it be kept under tight control. At least 12 proteins are known that do this. Three examples:

- **Factor H** removes Bb from the alternative pathway C3 convertase breaking the positive feedback loop.
- **Factor I** inactivates C3b.
- **C1 inhibitor** (**C1INH**) binds to sites on activated C1r and C1s shutting down their proteolytic activity. So when C1 is activated by antigen-antibody complexes, there is only a brief interval during which it can cleave C4 and C2 before it is **de**activated by C1INH.

Disorders of the Complement System

With so many proteins involved, it is not surprising that inherited deficiencies of one or another are sometimes encountered in humans. Four examples:

- **C3**. An inherited deficiency of C3 predisposes the person to frequent bouts of bacterial infections.
- **C2**. Curiously, immune complex disorders, not bacterial infections, are the main problem with a deficiency of C2 (or of one

of the other "early" components like C1q, C1r, C1s, or C4). This emphasizes the important role of the complement system in clearing away antigen-antibody complexes. A deficiency of C2 (or one of the other early components) is frequently found in patients with the autoimmune disorder systemic lupus erythematosus (SLE).

- **C9**. Another curiosity: most people who cannot make C9 have no more of a problem with bacterial infections than those who can. Laboratory studies suggest that the **C5b•6•7•8** complex by itself is able to lyze bacteria although not as efficiently as C9. And, in fact, a deficiency of **C8** is associated with a sharply-increased risk of bacterial meningitis.
- **C1INH**. A deficiency of C1INH produces **hereditary angioedema (HAE)**.

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15.4S: Inflammation

Inflammation is a response of a tissue to injury, often injury caused by invading pathogens. It is characterized by increased blood flow to the tissue causing increased temperature, redness, swelling, and pain.

A bacterial infection initiates inflammation through several interconnecting mechanisms:

- The "nonself" surface of bacteria allows the complement system to be activated through the "alternative pathway".
- Certain surface molecules of the bacteria, called Pathogen-Associated Molecular Patterns (PAMPs), bind to Toll-like receptors (TLRs) on or in a variety of leukocytes.

Mast Cells

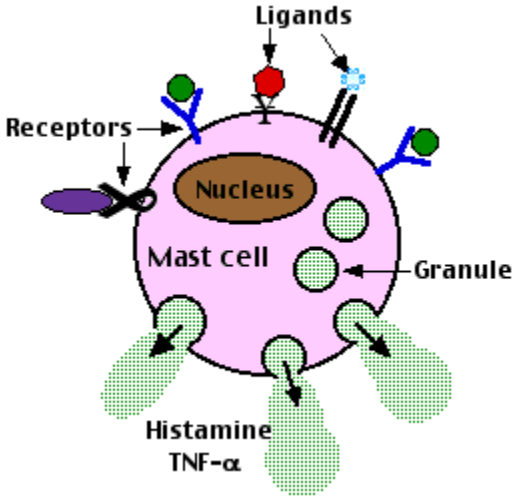


Figure 15.4.19.1 Mast cell

Mast cells are found in the tissues. Their cytoplasm is loaded with granules containing mediators of inflammation. Their surface is coated with a variety of receptors which, when engaged by the appropriate ligand, trigger exocytosis of the granules. Mast cells appear to be key players in the initiation of inflammation.

- Their Toll-like receptors trigger exocytosis when they interact with PAMPs like
  - the **lipopolysaccharide** (LPS or "endotoxin") of Gram-negative bacteria (TLR-4)
  - the peptidoglycan of Gram-positive bacteria (bind TLR-2)
- Their receptors for complement fragments trigger exocytosis when they bind C3a and C5a bacteria coated with C3b

Activated mast cells release literally dozens of potent mediators. Some immediately as they discharge their granules, some later as they synthesize them by new gene transcription. These mediators are active in either (or, in some cases, both)

- **recruiting** all the types of white blood cell to the site
  - monocytes that become macrophages when they leave the blood and enter the tissue
  - neutrophils
  - antigen-presenting dendritic cells
  - all kinds of lymphocytes:
    - B cells and T cells, leading to an adaptive immune response;
    - NK cells (an effector cell in innate immunity).
  - eosinophils
- **activating** many of these recruited cells to produce their own mediators of inflammation.

Tumor Necrosis Factor-alpha (TNF-α)

Large amounts of TNF-α are quickly released by stimulated mast cells. All the cells involved in inflammation have receptors for TNF-α and are activated by it to synthesize more on their own. This positive feedback quickly amplifies the response.

Tryptase

Tryptase is the most abundant protein released by mast cells. It is a serine protease. Like the mammalian enzyme trypsin, tryptase cleaves peptide bonds on the C-terminal side of arginines and lysines. It activates C3 of the complement system and probably supports inflammation in other ways as well.

Chemokines

These are **chemo**tactic cyto**kines**; that is, secreted proteins that attract other leukocytes into the area. Several have been identified.

Reactive Oxygen Species (ROS)

These are produced by activated phagocytes: macrophages and neutrophils. They are toxic for microorganisms but can also lead to tissue injury. ROS are described in detail on another page. Link to it.

Histamine

The granules of mast cells are loaded with histamine and their exocytosis releases this potent mediator. Histamine increases the blood flow to the area and the leakage of fluid and proteins from the blood into the tissue space. Thus the quick release of histamine produces the redness and swelling associated with inflammation.

Interleukin-1 (IL-1)

Macrophages, monocytes, and activated platelets are sources of this cytokine. IL-1 has both

- paracrine effects on cells in the vicinity
  - causing them to produce **tissue factor** and thus triggering the blood clotting cascade
  - stimulating the synthesis and secretion of a variety of other interleukins
  - helping to activate T cells and thus initiate an adaptive immune response
- endocrine (hormonal) effects as it is carried in the blood throughout the body
  - decreasing blood pressure
  - inducing **fever**.

IL-1 causes fever by stimulating the release of **prostaglandins**, which act on the temperature control center of the hypothalamus.

Inflammasomes

IL-1 is synthesized from a larger precursor that is cleaved by a caspase (caspase-1). Caspase-1 is part of a multi-protein complex in the cytosol of macrophages and neutrophils called an inflammasome. Inflammasomes are activated by several different products produced by invading bacteria. Some of these are first "seen" by toll-like receptors (TLRs) thus providing a link between the innate immune system and inflammation.

Bradykinin

Bradykinin is a nonapeptide (9 amino acids). It is synthesized by proteolytic cleavage of an inactive precursor (a kininogen) that is produced by the liver and circulates at all times in the blood (one of the alpha-globulins).

Bradykinin relaxes the smooth muscle walls of the arterioles lowering blood pressure and increasing blood flow to the tissue and makes the capillaries leakier, allowing blood components to enter the tissue space. These effects (like those of histamine) produce the redness, warmth, and swelling of inflammation.

The process:

- Hageman factor (also known as clotting factor XII [12]) normally circulates in the blood as inactive precursor.
- When tissue damage allows blood to escape into the tissue space, Hageman factor comes in contact with the collagens in the tissue space and becomes activated.
- Activated Hageman factor is a serine protease that cleaves an inactive precursor called prekallikrein into another serine protease — **kallikrein**.
- Kallikrein then cleaves kininogen forming bradykinin.

Bradykinin also

- stimulates the release of nitric oxide
- stimulates phospholipase to increase the production of **prostaglandins**
- mediates the closing of the ductus arteriosus when a baby is born
- plays a major role in the dangerous swelling associated with hereditary angioedema (HAE)

Prostaglandins and Leukotrienes

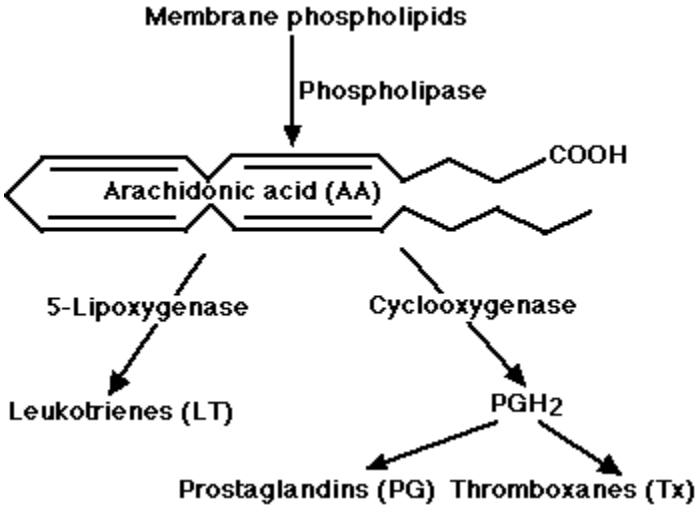


Figure 15.4.19.2 Prostaglandins

These potent mediators of inflammation are derivatives of **arachidonic acid** (**AA**) a 20-carbon unsaturated fatty acid produced from membrane phospholipids. The principal pathways of arachidonic acid metabolism are

- the **cyclooxygenase** (**COX**) pathway, which produces prostaglandin H_2 (**PGH_2**). PGH_2 serves as the substrate for two enzymatic pathways - one leading to the production of several **prostaglandins** (**PG**); the other leading to the production of **thromboxane** (**Tx**).
- the **5-lipoxygenase** pathway, which produces a collection of **leukotrienes** (**LT**)

Acute Inflammation: The Good Side of Inflammation

The acute inflammatory response to tissue damage is of great value. By

- isolating the damaged area
- mobilizing effector cells and molecules to the site
- in the late stages - promoting healing,

inflammation protects the body. Its importance is demonstrated by the problems people with inherited defects in components of

the process have with infections.

Some examples:

- a failure to produce reactive oxygen species (ROS) leads to **chronic granulomatous disease** (**CGD**)
- inherited defects in the ability to produce the later complement components (C5, C6, C7, C8, C9) increase the risk of certain infections.

Chronic Inflammation: The Bad Side of Inflammation

In chronic inflammation, the inflammatory response is out of proportion to the threat it is faced with or is directed against inappropriate targets. In the first case, the result can be more damage to the body than the agent itself would have produced.

Allergies and Autoimmune Diseases

All the many types of allergies and many of the autoimmune diseases are examples of inflammation in response to what should have been a harmless agent.

Some examples:

- Asthma
- Rheumatoid Arthritis (RA)
- Multiple Sclerosis (MS)
- Systemic Lupus Erythematosus (SLE)

In many of these cases, the problem is made worse by the formation of antibodies against self antigens or persistent antigens from smoldering infections. The antibodies complex with the antigens triggering the **complement system** with all its mediators of inflammation. The result: **immune complex disorders**.

Treating Inflammation

Inappropriate inflammation can be treated with

- **steroids** like the glucocorticoid **cortisol**
- **n**on**s**teroidal **a**nti-**i**nflammatory **d**rugs (NSAIDs) like aspirin and ibuprofen (e.g., Motrin®, Advil®).
- a number of therapeutic proteins produced by recombinant DNA technology.

NonSteroidal Anti-Inflammatory Drugs (NSAIDs)

The NSAIDs achieve their effects by blocking the activity of cyclooxygenases. The body produces two different forms of cyclooxygenase:

- COX-1, which is involved in pain, promoting clotting, and protecting the stomach
- COX-2, which is involved in the pain produced by inflammation.

In addition to reducing the fever and pain of inflammation, NSAIDs also inhibit clotting. They do this by interfering with the synthesis of thromboxane A_2 in platelets. This is the reason that aspirin is given to patients undergoing angioplasty. Many people take a baby aspirin a day in the hope of avoiding heart attacks. But regular use of NSAIDs has a downside: a tendency to develop ulcers in the stomach and duodenum. Most of the NSAIDs inhibit both COX-1 and COX-2. However, some newer drugs, the so-called **COX-2 inhibitors**, such as rofecoxib (Vioxx®) and celecoxib (Celebrex®) are much more active against COX-2 than COX-1.

COX-2 inhibitors are effective against inflammation and avoid damage to the GI tract. But, unfortunately, they increase the risk of blood clots — which can cause heart attacks and strokes — because they do not block the synthesis of thromboxane A_2 by platelets (which contain only COX-1). So people depending on NSAIDs for their heart protective effects must monitor any use of COX-2 inhibitors carefully.

In fact, because of the increased risk of heart attacks and strokes, the manufacturer of Vioxx® removed it from the market on 30 September 2004.

Therapeutic Proteins

Recombinant DNA and monoclonal antibody technology have produced some new therapies that are being enlisted in the battle against damaging inflammation.

- an IL-1 antagonist that binds and inactivates the IL-1 **receptor**.
- etanercept (Embrel®). A soluble version of the TNF-α **receptor**. It binds TNF-α preventing it from carrying out its many inflammatory actions. Potent but carries a severe risk of allowing infections to develop.
- **recombinant protein C**. To help the body dissolve the tiny clots that are triggered during inflammation.
- **Infliximab** (Remicade®). Binds to tumor necrosis factor-alpha (TNF-α). Shows promise against some inflammatory diseases such as rheumatoid arthritis (by blunting the activity of Th1 cells). Side-effects: can convert a latent case of tuberculosis into active disease; can induce the formation of autoantibodies (by promoting the development of Th2 cells).

In fact, all the more powerful anti-inflammatory agents (e.g., glucocorticoids) increase the risk of infection.

Sepsis and Septic Shock

On occasions, for reasons that are not entirely clear, the inflammatory response — usually to an infection by lipopolysaccharide (LPS)-bearing Gram-negative bacteria — spirals out of control progressing until it involves the entire body. This life-threatening development is called **sepsis**.

The circulatory system loses its integrity:

- There is a breakdown of the adherens junctions between the cells lining the capillaries allowing fluid to leak into the tissue spaces — edema.
- There is a breakdown in the control of blood clotting. What should have been a mechanism to help wall off an infected area and promote healing leads instead to a dangerous deposition of fibrin in small blood vessels throughout the body.

If these responses are massive, they can lead to **septic shock**

- a failure of many organs: lungs, kidneys, etc.
- a sharp drop in blood pressure
- death

Toxic Shock Syndrome

Some Gram-positive cocci can produce a similar condition, but here the eliciting agent is not LPS but a toxin liberated by the bacteria. In theory, anti-inflammatory agents should be useful in combating sepsis. But so far, only recombinant protein C has shown any promise (by inhibiting the formation of thrombin), and severe bleeding is a dangerous side-effect.

Inflammation and Cancer

Chronic inflammation is also a frequent cause of cancer. Liver cancer is often the sequel to years of inflammation caused by infection by hepatitis B and/or C viruses. Lung cancer often is the end stage of years of chronic inflammation caused by inhaled irritants, of which tobacco smoke is the most reliable. Cervical cancer can follow chronic infection and inflammation caused by papilloma viruses and chlamydiae. Chronic infection with the liver fluke *Opisthorchis viverrini* is responsible for many cases of bile duct cancer in Thailand and Laos. Bladder, colon, pancreas, stomach, and other cancers may similarly be the final stage of years of inflammation.

The strong link between chronic inflammation and cancer should not be surprising when you consider that the **reactive oxygen species** (ROS) liberated during inflammation are powerful DNA-damaging agents. There is increased mitosis in response to inflammation puts more cells at risk of mutations as they replicate their DNA during S phase. Apoptosis, the programmed death of damaged cells, is suppressed in inflamed tissue. So cells with precancerous genetic mutations, which should have committed

suicide, live on to grow into a full-blown cancer.

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15.4U: Asthma

The incidence of asthma in the United States (as well as in many other developed countries) has reached epidemic proportions. In the last two decades, the number of sufferers in the U. S. has doubled to more than 14 million people. In an attack of asthma, the bronchi become constricted, making it difficult to breathe in and — especially — out. The sufferer wheezes and coughs. Severe attacks can be life-threatening.

The Mechanism

An attack of asthma begins when an allergen is inhaled. The allergen binds to IgE antibodies — those that have binding sites for the allergen — on mast cells in the lungs. Binding triggers exocytosis of the mast cells with the release of **histamine** and **leukotrienes**. These substances cause the smooth muscle cells of the bronchi to contract narrowing the lumen of the bronchi. This is the **early phase**. They attract an accumulation of inflammatory cells — especially eosinophils — and the production of mucus. This is the **late phase**. With repeated attacks, the lining of the bronchi becomes damaged. Although asthma begins as an allergic response, in time attacks can be triggered by nonspecific factors like cold air, exercise, and tobacco smoke.

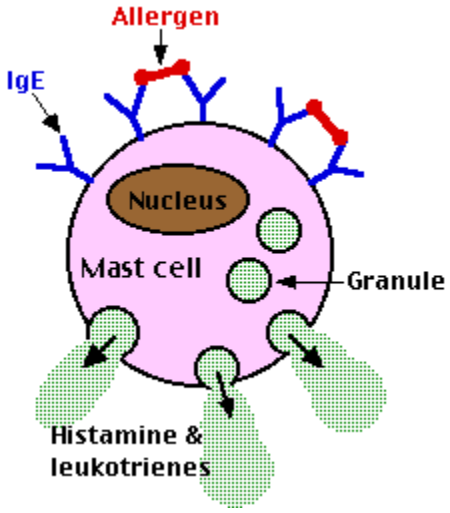


Figure 15.4.21.1: Mast cell

Some people are predispositioned to develop asthma

For reasons that are not yet understood, some people have a predisposition to respond to antigens by making antibodies of the IgE class. The trait tends to run in families suggesting a genetic component. These people are said to suffer from **atopy**. The T helper cells of atopic people are largely of the **Th2** type rather that Th1. And mice whose genes for a transcription factor (called "T-bet") used to make Th1 cells have been knocked out make fewer Th1 and more Th2 cells and suffer the lung changes typical of human asthma even though not exposed to any known allergen. Th2 helper cells help B cells make **IgE** antibodies by synthesizing interleukin 4 (**IL-4**) and interleukin 13 (**IL-13**), which promote class switching. They also release interleukin 5 (**IL-5**) which attracts eosinophils and other inflammatory cells to the site, producing the late phase of the response.

Asthma - a disease of developed countries

No one knows for certain. It is certainly **not** a matter of air pollution. Air pollution can trigger attacks of asthma, but some regions with heavily-polluted air have a much lower incidence of asthma than regions with relatively clean air. One intriguing possibility: sanitation and widespread childhood immunization may enable children to avoid the infections — especially viral — that stimulate the immune system to respond with Th1 helper cells rather than Th2 cells. Children in Europe that give positive DTH responses to tuberculin (a response mediated by Th1 cells) have lower rates of asthma than children who are negative in the tuberculin test. European children growing up on farms where they are exposed to high levels of bacteria and fungi associated with farm

animals have a lower incidence of asthma and atopy than their suburban peers. But children in tropical, undeveloped countries, who are often infected with parasitic worms, have **high** levels of Th2 cells and IgE but a very **low** incidence of asthma. Perhaps, then, a variety of chronic infections in childhood activate mechanisms (e.g., production of regulatory T cells) that suppress **all** inflammatory immune responses both Th1- and Th2-mediated.

Treatments

Beta-adrenergic agonists

- These drugs (albuterol is a popular example) mimic the action of adrenaline.
- They relax the smooth muscle of the bronchi.
- They may be inhaled or given by mouth.
- While useful in the early phase of an attack, they provide no protection against the longterm damage produced during the late phase.

Corticosteroids

- These drugs **reduce the inflammation** of the late phase of the response.
- They may be given in an inhaler (e.g., beclomethasone) or by mouth (e.g., prednisone)

Cromolyn sodium

Cromolyn sodium (disodium cromoglycate)

- **inhibits exocytosis of mast cells** thus blocking the release of histamine and leukotrienes
- is used mainly to **prevent** attacks (e.g., triggered by exercise) and is of no use in the early phase of an ongoing attack

Leukotriene inhibitors

Two types of leukotriene inhibitors received FDA approval in 1996.

- Zileuton (Zyflo®) blocks leukotriene **synthesis** by inhibiting the action of 5-lipoxygenase.
- Montelukast (Singulair®) blocks the leukotriene **receptors** on the surface of
  - smooth muscle cells
  - eosinophils

Possible future treatments still under investigation

- **Anti-IgE antibodies**. These interfere with the binding of IgE to mast cells. Omalizumab (Xolair®), a humanized monoclonal antibody produced by recombinant DNA technology, has been approved for use against allergic asthma (but carrying a "black-box" warning of the slight risk of its precipitating an anaphylactic reaction).
- Drugs that bind to **IL-13** keeping it from promoting IgE synthesis.
- Treatments that stimulate the production of **Th1 cells** by the immune system. Injections of a harmless mycobacterium (a relative of the TB bacillus) might do the trick. Th1 cells secrete **interferon-gamma** which is a powerful inhibitor of Th2 cells.

Some of these treatments are already in clinical trials.

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15.4V: AIDS

AIDS stands for **Acquired Immune Deficiency Syndrome**. It represents the late stages of infection by a retrovirus called **Human Immunodeficiency Virus** (**HIV**). The immune deficiency is caused by the loss of the **$CD4^+$ T cells** that are essential for both cell-mediated immunity and antibody-mediated immunity.

HIV - Human Immunodeficiency Viruses

They are retroviruses. There are two of them:

- **HIV-1** — the major cause of AIDS throughout the world;
- **HIV-2** — mostly found in West Africa.

Infection

HIV can only enter cells that express

- the transmembrane protein **CD4** found on helper T cells
- a second G protein-coupled "coreceptor" (GPCR) on these cells:
  - Strains of HIV (designated "**R5**") bind the coreceptor **CCR5**. These are the strains that are most infectious.
  - Strains of HIV (designated "**X4**") bind the coreceptor **CXCR4**.
  - Both strains usually coexist in an ongoing infection with X4 tending to dominate in the final stages of AIDS.

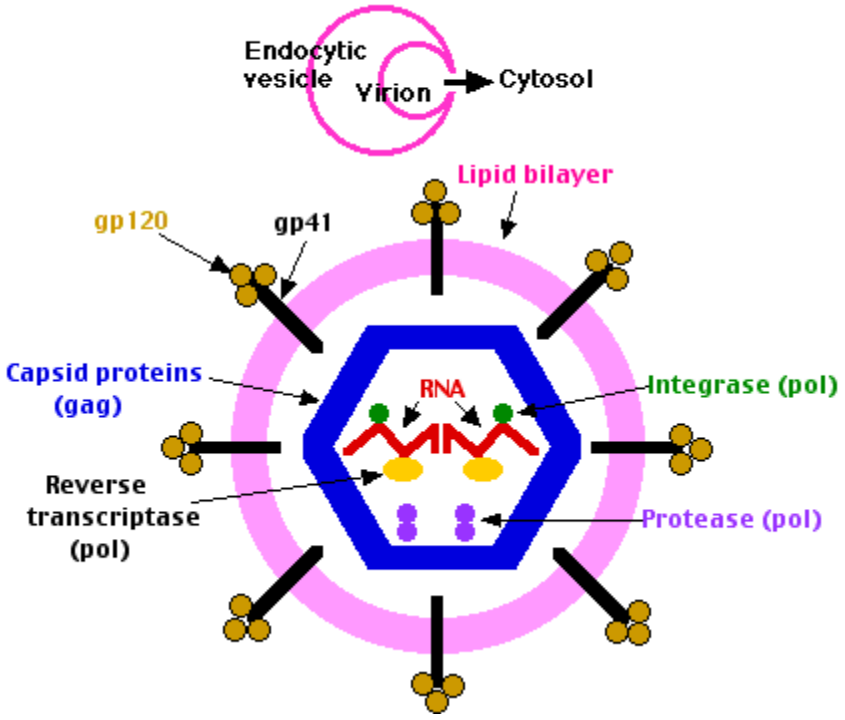


Figure 15.4.22.2 Endocytic vesicle

The virion binds to both CD4 and either coreceptor by means of molecules on its surface called glycoprotein 120 (**gp120**). The virion then is swept into the cell by receptor-mediated endocytosis. Fusion of the lipid membranes of the virion and the endosome - Endocytic vesicle, releases the contents of the virion into the cytosol. The fusion is mediated by gp41. When HIV infects a cell its molecules of **reverse transcriptase** and **integrase** are carried into the cell attached to the viral RNA molecules. The reverse transcriptase synthesizes **DNA** copies of the RNA. These enter the nucleus where the **integrase** catalyzes their insertion into the

DNA of the host's chromosomes. The HIV DNA is **transcribed** into fresh **RNA** molecules which reenter the cytosol where some are **translated** by host ribosomes. The **env** RNA is translated into molecules of the **envelope protein** (gp160). These pass through the endoplasmic reticulum and then the Golgi apparatus where they become glycosylated by enzymes of the host cell.

Proteases of the host cell then cut gp160 into

- **gp120** which sits on the surface of the virions (and is the target of most of the vaccines currently being tested).
- **gp41**, a transmembrane protein associated with gp120.
- the **gag and pol** genes are translated into a single protein molecule which is cleaved by the viral **protease** into
  - 6 different **capsid proteins**
  - the **protease**
  - **reverse transcriptase**
  - the **integrase**
- other RNA molecules become incorporated into fresh virus particles

Disease Transmission

HIV is present in body fluids especially blood and semen, especially in the early and late phases of the disease. Breaks or abrasions in mucous membranes and skin allow the virus in.

In North America, transmission occurs primarily

- between men when one ejaculates into the rectum (or mouth — the adenoids and tonsils are filled with **dendritic cells**) of the other
- among intravenous drug users who share needles
- in women who are the sexual partners of bisexual men or i.v. drug users
- in the newborn babies of these women
- in recipients of infected blood or blood products. This last category accounted for a devastating epidemic among hemophiliacs in the 1980s who unknowingly used HIV-contaminated preparations of **factor 8 (VIII)**. In some areas, 90% or more of the hemophiliacs developed AIDS. That risk, and the risk from blood transfusions, is now virtually zero because
  - all donated blood is now tested to see if the donor has been infected with HIV (as well as some other viruses)
  - plasma-derived preparations of factors 8 (VIII) and 9 (IX) are now treated with heat and/or solvents to destroy any viruses that might be present;
  - **recombinant factor 8 (VIII)** and **recombinant factor 9 (IX)** made by genetic engineering are now available.

Disease Progression

Infection by HIV produces three phases of disease:

- an early phase that
  - lasts about 2 weeks
  - is accompanied by fever, aches, and other flu-like symptoms
  - is accompanied by high levels of virus in the blood.
- a middle phase with these features:
  - lasts for months or even years
  - produces few, if any, symptoms
  - patient's blood contains few viruses, but contains **antibodies** to the virus which are the basis of the most common test for HIV infection
  - continuous infection, death, and replacement of $CD4^+$ T cells
- It is the late phase that is called **AIDS**. It has these features:
  - A rapid decline in the number of $CD4^+$ T cells. When these drop below about 350 per µl (normal is >1000), the patient's immunity is sufficiently weakened that **opportunistic infections** begin. These are infections caused by organisms that ordinarily do not cause disease symptoms in immunocompetent people. They include:
    - viruses, e.g., herpes simplex, herpes varicella-zoster, Epstein-Barr virus (EBV)
    - bacteria, e.g., *Mycobacterium tuberculosis*
    - fungi, e.g. ***Candida albicans*** (the cause of "thrush"), ***Pneumocystis jirovecii*** (causes pneumonia)

    - protozoans, e.g., Microsporidia
  - When the $CD4^+$ count drops below 200 per µl (mm^3), opportunistic infections become more severe and cancer (e.g., lymphoma, Kaposi's sarcoma) may develop. Untreated, these usually kill the patient within a year or so.

Treatment

In affluent countries, the progression of HIV disease has been markedly slowed by the use of **HAART** (= Highly Active AntiRetroviral Therapy). This refers to combined therapy with three or more drugs, e.g., two that target the reverse transcriptase and one that targets the viral protease.

Reverse Transcriptase Inhibitors

- Nucleoside analogs. Examples:
  - zidovudine (AZT)(Retrovir®)
  - lamivudine (Epivir®)
  - didanosine (Videx®)

  Each of these drugs "fools" the reverse transcriptase into incorporating it into the growing DNA strand which then halts further DNA synthesis.

- Other Reverse Transcriptase Inhibitors
- These drugs, e.g., efavirenz (Sustiva®) inhibit the enzyme by other mechanisms.

Protease Inhibitors

These block the viral protease so that the proteins needed for assembly of new viruses cannot be cleaved from the large protein precursor. Examples:

- indinavir (Crixivan®)
- saquinavir (Invirase®)
- ritonavir (Norvir®)

Fusion Inhibitors

Fusion of the virion membrane with the endosome membrane involves noncovalent binding between two segments of the **gp41** molecule designated HR1 and HR2. **Enfuvirtide** (Fuzeon®), a synthetic polypeptide containing 36 of the amino acids present in the HR2 segment, interferes with this process. It probably acts as a kind of competitive inhibitor, binding to HR1 thus preventing HR2 from binding HR1.

Integrase Inhibitors

Raltegravir (Isentress®), a drug that inhibits the HIV-1 integrase, has slowed disease progression in patients for whom other drugs were losing their effectiveness.

Inhibiting Coreceptor Binding

Several drugs — as well as some monoclonal antibodies — that block the binding of HIV to the coreceptors CCR5 and CXCR4 are being tested for safety and efficacy. Maraviroc®, a drug that binds to CCR5, has performed so well that it received FDA approval in 2007. People who have a mutation in their *CCR5* gene are resistant to infection. Early clinical trials of gene therapy in which a patient's normal *CCR5* gene is deliberately disrupted have shown promise.

Problems with drug treatment

Despite the great advances in slowing the progression of the disease, reversing at least for a time the symptoms of the late stages

of the disease and preventing the infection of babies born to infected mothers drug therapy has many drawbacks.

- The drugs are so expensive ($7,000 to $10,000 per year) that they not only drain resources in affluent countries but are simply unavailable in the many poor countries where the epidemic rages.
- They have many unpleasant side-effects (e.g., nausea, diarrhea, liver damage).
- They demand a very complicated dosing regimen: over a dozen pills a day (not counting those needed to cope with the accompanying opportunistic infections).
- They have to be continued even after active virus disappears because HIV-1 can integrate into the DNA of resting memory $CD4^+$ T cells as a **provirus** and emerge as active virus later.
- They often lose effectiveness as they select for the emergence of drug-resistant virions in the patient. This latter problem is particularly serious because of the speed at which mutations occur in HIV (as we shall now see).

Genetic Variability of HIV

Reverse transcription (RNA → DNA) lacks the proofreading capabilities of DNA replication or of normal transcription (DNA → RNA). Therefore errors, i.e., mutations, are frequent. Because of these,

- The population of viruses in a single patient becomes genetically more diverse as time goes by. This can lead to:
  - appearance of strains that invade other types of cells such as X4 strains that target T cells and strains that target cells of the brain, etc.
  - Development of resistance to the anti-viral drugs being used.
- New strains and subtypes of HIV-1 and HIV-2 arise in the human population.
  - These complicate the efforts to develop a vaccine against HIV
  - But as we shall now see that these have helped to unravel the origins of the disease.

Origin of HIV

Genome sequencing of different isolates of HIV-1 and HIV-2 shows that each is related to retroviruses that occur in primates in Africa. These are designated **simian immunodeficiency viruses** (**SIV**) although they do not cause immune deficiency (or any disease) in their natural host. However, on those occasions when a SIV accidentally infects a primate of a different species, it does cause disease in the new host. The human epidemic is one example.

- HIV-1 is most closely related to a SIV found in chimpanzees (***Pan troglodytes troglodytes***)
- HIV-2 is most closely related to a SIV that occurs in the sooty mangabey (***Cercocebus atys***).

Genome analysis also permits the construction of phylogenetic trees which reveal different clades of HIV just as such analysis reveals evolutionary relationship between species. The picture so far:

- HIV-1 appears to have infected humans on at least 4 different occasions giving rise to 4 clades: M, N, O, and P. Groups M and N appear to have jumped at separate times from chimpanzees to humans while O may have jumped from gorillas to humans. Except in parts of West Africa, most human cases are caused by members of Group M.
- HIV-2 appears to have jumped from sooty mangabeys to humans on at least 4 different occasions (there are 4 clades).
- How? These (and other) primates are often slaughtered for food and exposure to their blood and tissues is probably the route of transmission. In fact the chimpanzee SIV that gave rise to HIV-1 appears to be itself the product of recombination between two monkey SIVs that infected chimpanzees. (Chimps often eat monkeys.)

Just as with other evolutionary trees, one can also estimate from genome sequences the time of divergence of two branches. This evidence indicates that the Group M clade of HIV-1 invaded humans sometime very early in the 20th century.

But the worldwide epidemic of AIDS did not get its start until the 1980s. What took so long? An answer to that requires an appreciation of the way in which contagious diseases spread. Their rate of spread depends on:

- The **ease of transmission**. The transmissibility of HIV is **very low**. HIV is not like influenza or measles which spread like wildfire.
- The length of **time the host remains contagious**. Again, HIV is not like influenza or measles where the period of contagiousness is just a few days. For HIV, it can be years.
- The **number of susceptible contacts**; that is, the proximity of potential new hosts. For sexually-transmitted diseases (STDs),

that means the number of sexual contacts.

So diseases like HIV only smolder in **isolated** populations because they lack the density of susceptible contacts. In **crowded** populations, the equation changes. There has been a dramatic population shift from rural to urban areas in sub-Saharan Africa since 1950. In the case of STDs, the availability of multiple sexual contacts — perhaps accompanied by changing sexual mores — tips the scales. In any case, the major factor today in the spread of HIV is promiscuity, whether homosexual or heterosexual.

Prevention of AIDS

Vaccines

Many once-feared infectious diseases have been reduced or eliminated by the development of a vaccine to prevent the disease. Over two dozen experimental anti-HIV vaccines have been developed and clinical trials of some of these have been and are presently being undertaken. So far, the results have been disappointing. There are probably several reasons. Some of the vaccines attempt to induce antibodies, e.g., against the outer portion of the envelope protein (called **gp120**). But antibody-mediated immunity may not give adequate protection. The gene (***env***) encoding the envelope protein mutates too rapidly. The virus may be able to stay within cells out of the reach of circulating antibodies. High levels of antibodies (the basis of the most common test of infection) persist even while the disease pursues its inexorable course.

So other vaccines have been designed to favor the development of cell-mediated immunity; e.g., cytotoxic T cells.

Many of these are DNA vaccines, for example,

- a live virus such as canarypox (a harmless relative of smallpox) or an adenovirus which serves as a vector for introducing HIV genes (DNA)
- a mixture of plasmids encoding several HIV genes (e.g., *gag*, *pol*, and *env* of several HIV strains).

It is hoped that expression of these genes within the cells (e.g., muscle) of the subject will induce a protective immune response, but to date there has been no success.

Behavior

Because HIV transmission is so difficult, changing behavior could go a long way toward stopping the epidemic.

- Reducing the number of sexual partners.
- If injecting drugs cannot be stopped, then using sterile needles (thus not sharing them) would prevent infection.
- Using condoms and/or other (e.g., chemical) barriers to prevent contact with infectious semen.

In the words of Anthony S. Fauci, Director of the National Institute of Allergy and Infectious Diseases, "Unlike microbial scourges, such as malaria and tuberculosis (among many others), for which there is very little that people can do to prevent infection, **HIV infection in adults is entirely preventable by behavior modification**".

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15.4W: Vaccines

Active Immunization or Vaccination

The terms vaccination and vaccine derive from the work of Edward Jenner who, over 200 years ago, showed that inoculating people with material from skin lesions caused by cowpox (*L. vaccinus*, of cows) protected them from the highly contagious and frequently fatal disease smallpox. Since Jenner's time, the term has been retained for any preparation of dead or weakened pathogens, or their products, that when introduced into the body, stimulates the production of protective antibodies or T cells without causing the disease. In molecular terms, the goal is to introduce harmless antigen(s) with epitopes that are also found on the pathogen.

Vaccination is also called **active** immunization because the immune system is stimulated to develop its own immunity against the pathogen. **Passive immunity**, in contrast, results from the injection of antibodies formed by another animal (e.g., horse, human) which provide immediate, but temporary, protection for the recipient.

Kinds of Vaccines

Killed whole organisms

In this relatively crude approach, the vaccine is made from the entire organism, killed to make it harmless. The typhoid and cholera vaccines are examples.

Attenuated organisms

Here, the organism has been cultured so as to reduce its pathogenicity, but still retain some of the antigens of the virulent form. The Bacillus Calmette-Guérin (BCG) is a weakened version of the bacterium that causes tuberculosis in cows. BCG is used as a vaccine against tuberculosis in many European countries but is rarely used in the U.S.

Toxoids

In some diseases, **diphtheria** and **tetanus** are notorious examples, it is not the growth of the bacterium that is dangerous, but the protein toxin that is liberated by it. Treating the toxin with, for example, formaldehyde, denatures the protein so that it is no longer dangerous, but retains some epitopes on the molecule that will elicit protective antibodies.

Surface molecules

Antibodies are most likely to be protective if they bind to the surface of the invading pathogen triggering its destruction. Several vaccines employ purified surface molecules:

- Influenza vaccine contains purified hemagglutinins from the viruses currently in circulation around the world.
- The gene encoding a protein expressed on the surface of the hepatitis B virus, called hepatitis B surface antigen or **HBsAg**, can now be expressed in *E. coli* cells and provides the material for an effective vaccine. Hepatitis B infection is strongly associated with the development of liver cancer. Here then is a vaccine against a cancer.
- The genes encoding the capsid proteins of 9 strains of human papilloma virus (**HPV**) can be expressed in yeast and the resulting recombinant proteins are incorporated in a vaccine (Gardasil 9®). Because infection with some of these strains of HPV can lead to cervical cancer, here is another vaccine against cancer.
- Some 80 different strains of ***Streptococcus pneumoniae*** cause pneumonia in humans. They differ in the chemistry of the polysaccharide capsule that surrounds them and makes it difficult for phagocytes to engulf them by endocytosis. One current vaccine consists of tiny amounts of the purified capsular polysaccharides of the 23 most common and/or dangerous strains.

Inactivated virus

Like killed bacterial vaccines, these vaccines contain whole virus particles that have been treated (again, often with formaldehyde) so that they cannot infect the host's cells but still retain some unaltered epitopes. The Salk vaccine for polio (IPV) is an example.

Attenuated virus

In these vaccines, the virus can still infect but has been so weakened that it is no longer dangerous. The measles, mumps, and rubella ("German measles") vaccines are examples. The Sabin oral polio vaccine (OPV) is another example. It has advantages over the Salk vaccine in that

- it is given by mouth rather than by injection;
- the viruses it contains can spread to the other members of the vaccinee's family thus immunizing them as well.

It has the disadvantage that on rare occasions one of the strains in the vaccine regains full virulence and causes the disease. For this reason, the Salk vaccine has once again become the preferred vaccine worldwide.

Example: A new method of attenuation

The various attenuated-virus vaccines in current use were developed by rather hit-or-miss methods. However, scientists have been working on a technology exploiting the phenomenon of **codon bias** - that may make possible the rational development of safer vaccines.

One group, at Stony Brook University (see J.R. Coleman *et al.*, *Science*, 27 June 2008), has engineered polio virus with hundreds of mutations in the genes encoding its capsid protein. However, every one of these is a "silent" mutation; that is, it simply changes the codon for the amino acid to a different codon for the same amino acid. When they created polio viruses in which pairs of new codons were ones that the wild polio virus avoids using (because its human host does), they found that the new viruses were far less infectious that the original. But note, that this procedure did not introduce any change in the amino acid sequence of the capsid protein. So one would expect that all the epitopes recognized by the immune system would be unchanged. And, indeed, they went on to show that mice immunized with the synthetic virus were protected from disease caused by the wild virus.

As mentioned above, one of problems associated with the attenuated live virus polio vaccine (Sabin) is the rare back mutation to full virulence. Such back mutation in these engineered viruses would be extremely unlikely considering the hundreds of silent mutations that would have to be reversed.

Summary Table: Here is a table describing some of the most widely-used vaccines used in humans.

Disease Agent	Preparation	Notes
Diphtheria	Toxoid	Often given in a single preparation **DTaP** (Daptacel®) for infants and young children; **Tdap** for teenagers and adults (the lower case letters signify the smaller amounts of the diphtheria and pertussis antigens in Tdap).
Tetanus	Toxoid	
Pertussis	Killed bacteria ("P") or their purified components (acellular pertussis = "aP")	
Polio	Inactivated virus	Inactivated polio vaccine: IPV (Salk)
	Attenuated virus	Oral polio vaccine; OPV (Sabin) Both vaccines trivalent (types 1, 2, and 3)
Hepatitis A	Inactivated virus	HAVRIX® and VAQTA®; also available in single shot with HBsAg (Twinrix®)
Hepatitis B	Protein (HBsAg) from the surface of the virus	Made by recombinant DNA technology

Disease Agent	Preparation	Notes
Rotavirus	Attenuated virus (Rotarix®) or 5 strains of the virus (RotaTeq®)	to prevent this serious diarrheal disease in infants
Human Papilloma Virus (HPV)	Protein from the capsid of 9 strains of the virus	Gardasil 9®; made by recombinant DNA technology
Diphtheria, tetanus, pertussis, polio, and hepatitis B	Uses acellular pertussis and IPV (Salk)	Pediarix®; combination vaccine given in 3 doses to infants
Diphtheria, tetanus, pertussis, polio, and *Hemophilus influenzae* type b (Hib)	Uses acellular pertussis and IPV (Salk)	Pentacel®; combination vaccine given in 4 doses to infants
Measles	Attenuated virus	Often given as a mixture (MMR) Do **not** increase the risk of autism. (Nor do any vaccines containing thimerosal as a preservative.)
Mumps	Attenuated virus	
Rubella	Attenuated virus	
Chickenpox (Varicella)	Attenuated varicella-zoster virus (VZV)	Also available combined with MMR ("MMRV" or ProQuad®)
Cholera	Killed bacteria	Three oral vaccines available
Influenza	Hemagglutinins	Contains hemagglutinins from the type A and type B viruses recently in circulation
	Attenuated virus	FluMist® — contains weakened viruses of the type B and two type A strains recently in circulation
Pneumococcal infections	Capsular polysaccharides	A mixture of the capsular polysaccharides of 23 common types. Works poorly in infants.
	13 capsular polysaccharides conjugated to protein ("PCV13")	Mobilizes helper T cells; works well in infants.
Meningococcal disease	4 polysaccharides conjugated to protein	To prevent outbreaks among new groups of young adults, e.g., college freshmen, military recruits
Hemophilus influenzae, type b (Hib)	Capsular polysaccharide conjugated to protein	Prevents meningitis in children
Rabies	Inactivated virus	Vaccine prepared from human diploid cell cultures (HDCV) or chick embryo cells (PCECV)
Smallpox	Vaccinia virus	Despite the global eradication of smallpox, is used to protect against a possible bioterrorist attack
Anthrax	Extract of attenuated bacteria	Primarily for veterinarians and military personnel
Typhoid	Three types are available: 1. killed bacteria 2. live, attenuated bacteria (oral) 3. polysaccharide conjugated to protein	
Yellow fever	Attenuated virus	

Disease Agent	Preparation	Notes
Tuberculosis	Attenuated bacteria (BCG)	Rarely used in the U.S.

Some of the Triumphs of Vaccination

The greatest triumph is the eradication of smallpox from the planet, with no naturally-occurring cases having been found since 1977. "Naturally-occurring" because one case (fatal) occurred later following the accidental release of the virus in a laboratory. As far as the public knows, smallpox virus now exists only in laboratories in the U.S. and Russia. There is currently a vigorous debate as to whether these should be destroyed. If smallpox ever should get back out into the environment, the results could be devastating because smallpox vaccination is no longer given and so the population fully susceptible to the disease grows year by year.

A program to try to eliminate polio from the world is now underway. Except for cases caused by OPV, the disease has now been eliminated from the Western hemisphere. Outbreaks of polio still occur in Africa, the Indian subcontinent, and parts of the Near East. Table 1 compares the number of cases of illness in the U.S. in a representative year (either before a vaccine was available or before it came into widespread use) with the number of cases reported in 1994.

Table 1

Disease	Total cases	Year	Cases in 1994	% Change
Diphtheria	206,939	1921	2	-99.9%
Measles	894,134	1941	963	-99.9%
Mumps	152,209	1968	1537	-99.9%
Pertussis	265,269	1934	4617	-99.9%
Poliomyelitis*	21,269	1952	0	-100%
Rubella	57,686	1969	227	-99.9%
Tetanus	1,560	1923	51	-99.9%

*Since 1979, an average of 8 cases of poliomyelitis have occurred in the U.S. each year that are acquired from the vaccine (OPV, the Sabin vaccine) itself. For this reason, the "killed" virus vaccine (IPV, the Salk vaccine) is being reintroduced. As of June 17, 1999, it is recommended that in the future all children receive 4 doses of the Salk vaccine and — except in special circumstances — none of the Sabin vaccine.

Problems of Vaccine Development

With so many triumphs, why haven't vaccines eliminated other common diseases such as **malaria** and **HIV-1** infection?

One problem is that experimental vaccines often elicit an immune response that does not actually protect against the disease. Most vaccines preferentially induce the formation of antibodies rather than **cell-mediated immunity**. This is fine for those diseases caused by

- toxins (diphtheria, tetanus)
- extracellular bacteria (pneumococci)
- even viruses that must pass through the blood to reach the tissues where they do their damage (polio, rabies)

But viruses are intracellular parasites, out of the reach of antibodies while they reside within their target cells. They must be attacked by the cell-mediated branch of the immune system, such as by cytotoxic T lymphocytes (CTLs). Most vaccines do a poor job of eliciting cell-mediated immunity (CMI).

Example:

Much of the early and so far unsuccessful work on anti-HIV-1 vaccines has focused on the antibody response of the test animal. Antibodies may have a role in preventing infection or minimizing its spread, but cell-mediated responses will probably turn out to

be far more important. Certainly there are thousands of patients dying of AIDS despite their high levels of anti-HIV-1 antibodies. The most widespread test for HIV-1 infection does not detect the presence of the virus but the presence of antibodies against the virus.

DNA Vaccines

With DNA vaccines, the subject is not injected with the antigen but with DNA encoding the antigen.

The DNA is incorporated in a plasmid containing

- DNA sequences encoding one or more protein antigens or, often, simply epitopes of the complete antigen(s);
- DNA sequences incorporating a promoter that will enable the DNA to be efficiently transcribed in the human cells.
- Sometimes DNA sequences encoding costimulatory molecules and sequences that target the expressed protein to specific intracellular locations (e.g., endoplasmic reticulum) are included as well.

The DNA vaccine can be injected into a muscle just as conventional vaccines are. In contrast to conventional vaccines, DNA vaccines elicit cell-mediated as well as antibody- mediated immune responses.

The cell-mediated response

- The plasmid is taken up by an antigen-presenting cell (**APC**) like a dendritic cell.
- The gene(s) encoding the various components are transcribed and translated.
- The protein products are degraded into peptides.
- These are exposed at the cell surface nestled in class I histocompatibility molecules where
- they serve as a powerful stimulant for the development of cell-mediated immunity.

The antibody-mediated response

- If the plasmid is taken up by other cells (e.g. muscle cells), the proteins synthesized are released and can be engulfed by antigen-presenting cells (including B cells).
- In this case, the proteins are degraded in the class II pathway and presented to helper T cells.
- These secrete lymphokines that aid B cells to produce antibodies.

So far, most of the work on DNA vaccines has been done in mice where they have proved able to protect them against **tuberculosis**, SARS, smallpox, and other intracellular pathogens. In addition, more than a dozen different DNA vaccines against HIV-1 the cause of AIDS are in clinical trials.

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15.5: EXCRETION

- Topic hierarchy

15.5A: Human Kidneys
15.5B: Vertebrate Kidneys
15.5C: Urea Cycle

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15.5A: Human Kidneys

The human kidneys are two bean-shaped organs, one on each side of the backbone. They represent about 0.5% of the total weight of the body, but receive 20–25% of the total arterial blood pumped by the heart. Each contains from one to two million **nephrons**.

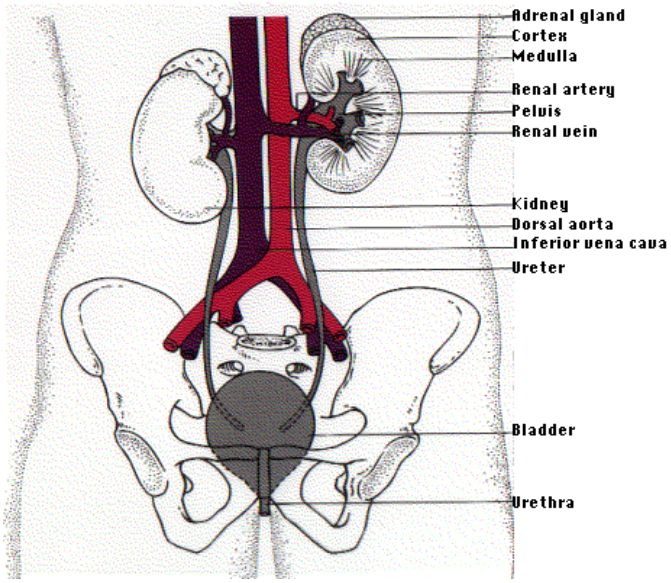


Figure 15.5.1.1 Human kidneys

The Nephron

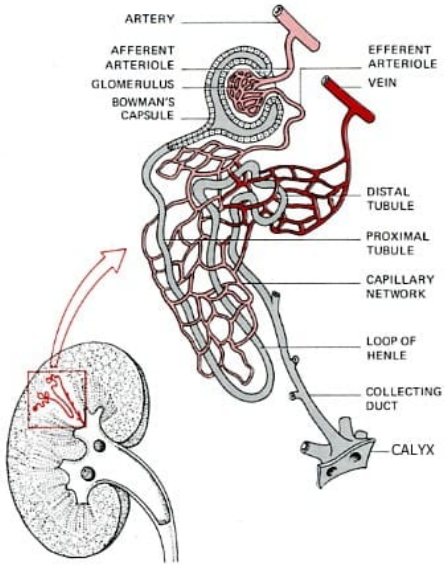


Figure 15.5.1.2 Nephrons

The nephron is a tube closed at one end and open at the other. It consists of:

- **Bowman's capsule**. Located at the closed end, the wall of the nephron is pushed in forming a double-walled chamber.
- **Glomerulus**. A capillary network within the Bowman's capsule. Blood leaving the glomerulus passes into a second capillary network surrounding the proximal tubule.
- **Proximal convoluted tubule**. Coiled and lined with cells carpeted with microvilli and stuffed with mitochondria.

- **Loop of Henle**. It makes a hairpin turn and returns to the distal convoluted tubule.
- **Distal convoluted tubule**, which is also highly coiled and surrounded by capillaries.
- **Collecting duct**. It leads to a calyx, one of many small chambers from which urine drains into the pelvis of the kidney from where it flows through a ureter to the bladder and, periodically, on to the outside world.

The Bowman's capsules are packed in the cortex of the kidney, the tubules and collecting ducts descend into the medulla.

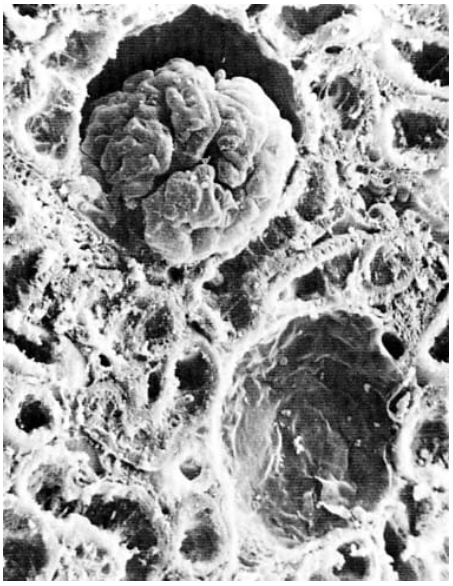


Figure 15.5.1.3 Cortex of a mouse kidney courtesy of Keith R. Porter

The image above shows a cut section of the cortex of a mouse kidney as seen under the scanning electron microscope. Near the top (center) can be seen a Bowman's capsule with its glomerulus. Directly beneath is another Bowman's capsule with its glomerulus removed. The remainder of the field shows the lumens of both proximal and distal tubules as they have been cut at various angles.

Formation of Urine

The nephron makes urine by filtering the blood of its small molecules and ions and then reclaiming the needed amounts of useful materials. Surplus or waste molecules and ions are left to flow out as urine. In 24 hours the kidneys reclaim: ~1,300 g of NaCl, ~400 g $NaHCO_3$, ~180 g glucose and almost all of the 180 liters of water that entered the tubules. Blood enters the glomerulus under pressure. This causes water, small molecules (but not macromolecules like proteins) and ions to filter through the capillary walls into the **Bowman's capsule**. This fluid is called **nephric filtrate**. As the table shows, it is simply blood plasma minus almost all of the plasma proteins. Essentially it is no different from interstitial fluid.

Table 1: Composition of plasma, nephric filtrate, and urine (each in g/100 ml of fluid). These are representative values. The values for salts are especially variable, depending on salt and water intake.

Component	Plasma	Nephric Filtrate	Urine	Concentration	% Reclaimed
Urea	0.03	0.03	1.8	60X	50%
Uric acid	0.004	0.004	0.05	12X	91%
Glucose	0.10	0.10	None	-	100%
Amino acids	0.05	0.05	None	-	100%
Total inorganic salts	0.9	0.9	<0.9–3.6	<1–4X	99.5%
Proteins and other macromolecules	8.0	None	None	-	-

Figure 15.5.1.4 Urine formation process

- Nephric filtrate collects within the Bowman's capsule and then flows into the **proximal tubule**.
- Here all of the **glucose** and **amino acids**, >90% of the uric acid, and ~60% of inorganic **salts** are reabsorbed by active transport.
  - The active transport of Na^+ out of the **proximal tubule** is controlled by angiotensin II.
  - The active transport of phosphate (PO_4^{3-}) back into the blood is regulated (suppressed) by both the parathyroid hormone and fibroblast growth factor 23 (FGF-23).
- As these solutes are removed from the nephric filtrate, a large volume of the water follows them by osmosis (80–85% of the 180 liters deposited in the Bowman's capsules in 24 hours).
- As the fluid flows into the descending segment of the **loop of Henle**, water continues to leave by osmosis because the interstitial fluid is very hypertonic. This is caused by the active transport of Na^+ out of the tubular fluid as it moves up the ascending segment of the loop of Henle.
- In the **distal tubules**, more sodium is reclaimed by active transport, and still more water follows by osmosis.
- Final adjustment of the sodium and water content of the body occurs in the **collecting ducts**.

Sodium

Although 97% of the sodium has already been removed, it is the last 3% that determines the final balance of sodium and hence water content and blood pressure in the body. The reabsorption of sodium in the **distal tubule** and the collecting ducts is closely regulated by the synergistic action of the hormones **vasopressin** and **aldosterone**.

Water

- The hypertonic interstitial fluid surrounding the collecting ducts provides a high osmotic pressure for the removal of water.
- Transmembrane channels made of proteins called **aquaporins** are inserted in the plasma membrane greatly increasing its permeability to water. When open, an aquaporin channel allows 3 billion molecules of water to pass through each second.
- Insertion of aquaporin-2 channels requires signaling by **vasopressin** (also known as arginine vasopressin [AVP] or the **antidiuretic hormone** [ADH]).
  - Vasopressin binds to receptors (called **V2 receptors**) on the basolateral surface of the cells of the collecting ducts.
  - Binding of the hormone triggers a rising level of **cAMP** within the cell.
  - This **"second messenger"** initiates a chain of events culminating in the insertion of **aquaporin-2** channels in the apical surface of the cell.
  - The release of **vasopressin** (from the posterior lobe of the pituitary gland) is regulated by the osmotic pressure of the blood.
- Anything that dehydrates the body, such as perspiring heavily,
  - increases the osmotic pressure of the blood
  - turns on the **vasopressin → V2 receptors → aquaporin-2** pathway.

  The result:
  - As little as 0.5 liter/day of urine may remain of the original 180 liters/day of nephric filtrate.
  - The concentration of salts in the urine can be as much as four times that of the blood. (But not high enough to enable humans to benefit from drinking sea water, which is saltier still.)
- If the blood should become too dilute (as would occur after drinking a large amount of water),
  - Vasopressin secretion is **inhibited**.
  - The aquaporin-2 channels are taken back into the cell by endocytosis.
  - The result: a large volume of watery urine is formed (with a salt concentration as little as one-fourth of that of the blood).

Diabetes insipidus

This disorder is characterized by excretion of large amounts of a watery urine (as much as 30 liters - about 8 gallons each day and unremitting thirst.

It can have several causes:

- Insufficient secretion of **vasopressin**.
- Inheritance of two mutant genes for the **vasopressin receptor** (V2) [in females; because the gene is X-linked, only one does it for males].

- Inheritance of one (for dominant mutations) or two (for recessive versions) mutant genes for **aquaporin-2**.

Liddle's Syndrome

The most obvious effect of this rare inherited disorder is extremely high blood pressure (hypertension). It is caused by a single mutant allele (therefore the syndrome is inherited as a dominant trait) encoding the aldosterone-activated **sodium channel** in the **collecting ducts**. The defective channel is always "on" so too much Na^+ is reabsorbed and too little is excreted. The resulting elevated osmotic pressure of the blood produces hypertension.

Tubular Secretion

Although urine formation occurs primarily by the filtration-reabsorption mechanism described above, an auxiliary mechanism, called tubular secretion, is also involved. The cells of the tubules remove certain molecules and ions from the blood and deposit these into the fluid within the tubules.

Example: Excess hydrogen ions (H^+) are combined with ammonia (NH_3) to form ammonium ions (NH_4^+) and transported to the cells of the collecting ducts. Here the NH_4^+ dissociates back into ammonia and H^+. Both are then secreted into the fluid within the collecting ducts (the protons by active transport).

Tubular secretion of H^+ is important in maintaining control of the pH of the blood. When the pH of the blood starts to drop, more hydrogen ions are secreted. If the blood should become too alkaline, secretion of H^+ is reduced. In maintaining the pH of the blood within its normal limits of 7.3–7.4, the kidney can produce a urine with a pH as low as 4.5 or as high as 8.5. Excess potassium ions (K^+) are also disposed of by tubular secretion.

The Kidney and Homeostasis

While we think of the kidney as an organ of excretion, it is more than that. It does remove wastes, but it also removes normal components of the blood that are present in greater-than-normal concentrations. When excess water, sodium ions, calcium ions, potassium ions, and so on are present, the excess quickly passes out in the urine. On the other hand, the kidneys step up their reclamation of these same substances when they are present in the blood in less-than-normal amounts. Thus the kidney continuously regulates the chemical composition of the blood within narrow limits. The kidney is one of the major homeostatic devices of the body.

Hormones of the Kidneys

The human kidney is also an endocrine gland secreting two hormones:

- **Erythropoietin** (**EPO**)
- **Calcitriol** ($1,25[OH]_2$ Vitamin D_3), the active form of vitamin D

as well as the enzyme **renin**.

The Artificial Kidney

The artificial kidney uses the principle of dialysis to purify the blood of patients whose own kidneys have failed.

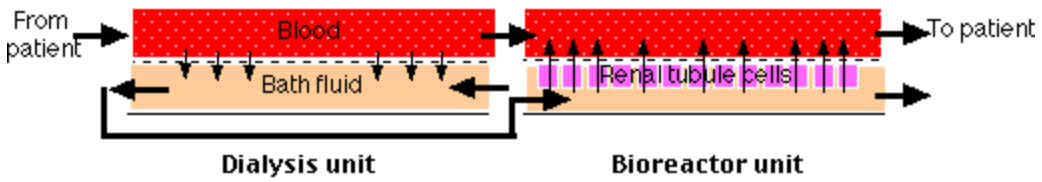


Figure 15.5.1.5 Artificial kidney

The **left** portion of the figure ("Dialysis unit") shows the mechanism used today in artificial kidneys. Small molecules like urea are removed from the blood because they are free to diffuse between the blood and the bath fluid, whereas large molecules (e.g., plasma proteins) and cells remain confined to the blood. The bath fluid must already have had essential salts added to it to prevent the dangerous loss of these ions from the blood. Note that blood and bath fluid flow in opposite directions across the dialysis membrane. This "counter-current" exchange maintains a diffusion gradient through the entire length of the system. An anticoagulant is added to the blood so it will not clot while passing through the machine. The anticoagulant is neutralized as the blood is returned to the patient.

Artificial kidneys have proved of great benefit in helping patients of acute kidney malfunction survive the crisis until their own kidneys resume operation. They have also enabled people suffering from chronic kidney failure to remain alive, though at an enormous expense of time (often three sessions of 6 or more hours per week), money, and psychological well-being. Furthermore, although dialysis does a good job at removing wastes, it cannot perform the other functions of the kidney:

- providing precise homeostatic control over the concentration of such vital ingredients as glucose and Na^+
- secreting its hormones

An Artificial Kidney of the Future?

In an attempt to solve these problems, a research team at the University of Michigan is experimenting with adding a "Bioreactor unit" (above) to the dialysis unit. The bioreactor consists of many hollow, porous tubes on the inner wall of which is attached a monolayer of **proximal tubule cells** (derived from pigs). The dialysis bath fluid passes through the lumen of the tubes where molecules and ions can be picked up by the apical surface of the cells. Discharge of essential molecules and ions (as well as hormones) at the basolateral surface of the cells places these materials back in the blood (just as the proximal tubule cells in the nephron normally do). So far, all the testing has been done using dogs, but the results seem promising.

Kidney Transplants

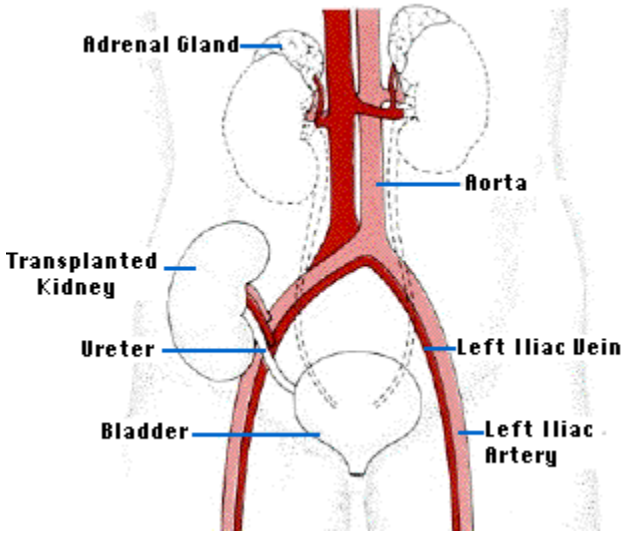


Figure 15.5.1.6 Kidney transplant

The ideal alternative to long-term dialysis is transplantation of a new kidney. The operation is technically quite easy. The recipient's diseased kidneys are usually left in place, but the renal arteries and veins are tied off except for the branches supplying the adrenal glands. The major problems is the shortage of donors suitably matched for histocompatibility molecules so as to

avoid the problem of graft rejection by the recipient's immune system - that unless the donor and recipient are identical twins - "sees" the kidney as "foreign".

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15.5B: Vertebrate Kidneys

All vertebrates have kidneys. Like the human kidney, they are made up of many nephrons. However, there are differences in the structure and functioning of various vertebrate kidneys that adapt them to the environment in which the animals live.

Freshwater Vertebrates

All animals that live in fresh water must cope with a continual inflow of water from their hypotonic environment. In order to maintain homeostasis of its extracellular fluid (ECF), the freshwater fish must excrete this excess water. Contraction of its heart (powered by ATP) provides the pressure to force the water, small molecules, and ions into the **glomerulus** as nephric filtrate. The essential ingredients are then reclaimed by the tubules, returning to the blood in the capillaries surrounding the tubules. The blood in these capillaries comes from the glomerulus (as in humans) and also from the **renal portal veins** which drain the posterior part of the fish's body.

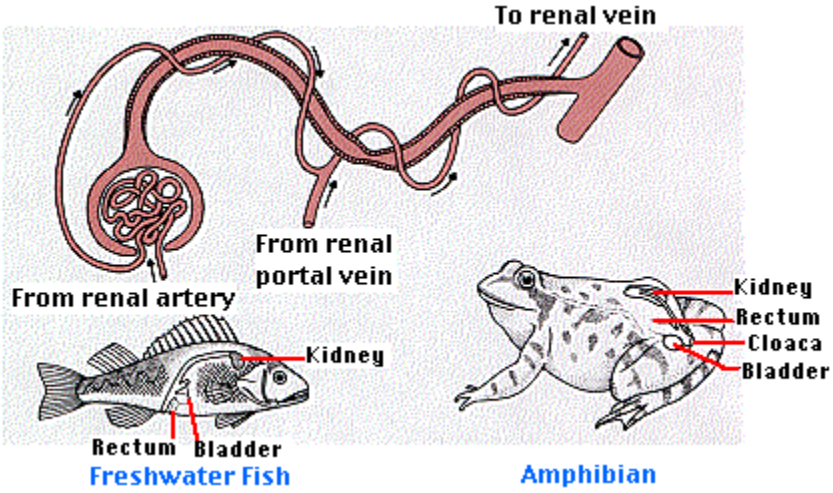


Figure 15.5.2.1 Freshwater kidneys

After solute reabsorption is complete, the urine is little more than water. Most of the nitrogenous wastes (including large amounts of ammonia, NH_3) leave by diffusion out of the gills. So, the kidney is mostly a device for maintaining water balance in the animal, rather than an organ of excretion.

Amphibians

The amphibian kidney also functions chiefly as a device for excreting excess water. The permeable skin of the frog provides an easy route for the fresh water of its pond to enter by osmosis. But, as their name suggests, amphibians also spend time on land. Then the problem is to conserve water, not eliminate it.

The frog adjusts to the varying water content of its surroundings by adjusting the rate of filtration at the glomerulus. When blood flow through the glomerulus is restricted, a **renal portal** system is present to carry away materials reabsorbed through the tubules. The frog is also able to use its urinary bladder to aid water conservation. When in water, the frog's bladder quickly fills up with a hypotonic urine. On land, this water is reabsorbed into the blood helping to replace water lost through evaporation through the skin. The reabsorption is controlled by a hormone similar to mammalian ADH.

Lizards and Snakes

Many reptiles live in dry environments (e.g., rattlesnakes in the desert). Among the many adaptations to such environments is their ability to convert waste nitrogen compounds into **uric acid**. Uric acid is quite insoluble and so can be excreted using only a small amount of water. Thus we find that reptile glomeruli are quite small and, in fact, some reptiles have no glomeruli at all.

Those with glomeruli filter just enough fluid to wash the uric acid, which is secreted by the tubules, into the **cloaca**. Most of this moisture is reabsorbed in the cloaca. Emptying the cloaca deposits feces (brown) and uric acid (a white paste). The cloaca is a chamber through which the feces and the gametes, as well as urine, pass on the way to the outside. The name comes from the Latin word for sewer. These water conservation mechanisms can allow the reptile to forgo drinking water. The water content of its food plus the water produced by cellular respiration is usually sufficient.

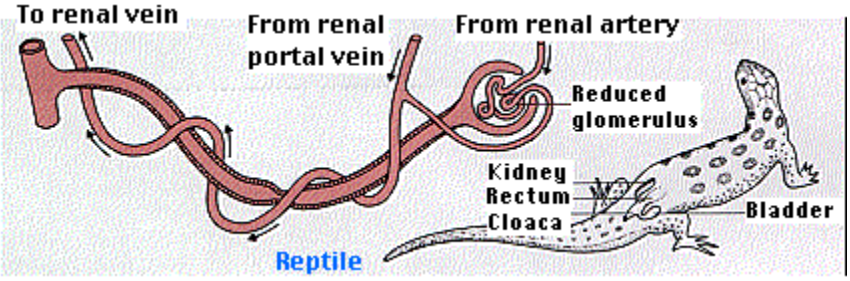


Figure 15.5.2.2 Reptile kidneys

Birds

Bird kidneys function like those of reptiles (from which they are descended). Uric acid is also their chief nitrogenous waste. Most birds have a limited intake of fresh water. However, they need filter only enough to wash a slurry of uric acid into the cloaca where enough additional water is reclaimed to convert the uric acid into a semisolid paste. It is the whitish material that pigeons leave on statues.

Mammals

All mammals share our use of urea as their chief nitrogenous waste. Urea requires much more water to be excreted than does uric acid. Mammals produce large amounts of nephric filtrate but are able to reabsorb most of this in the tubules. But even so, humans lose several hundred ml each day in flushing urea out of the body. Some mammals have more efficient kidneys than ours. The kangaroo rat of the desert can produce a urine 17 times more concentrated that its blood. (The best we can do is 3-4 times as concentrated.) The efficiency of the kangaroo rat kidney enables it to survive without drinking water — simply depending on the water content of its food and that produced by cellular respiration.

We like to think of ourselves as highly advanced. Why don't we have kidneys as efficient as those of the reptiles and birds? It is the luck of our inheritance. The line of vertebrate evolution that produced the mammals split off before the evolution of the **diapsids** whose ability to convert nitrogenous wastes into uric acid was passed on to all their descendants, including the lizards, snakes, and birds.

Marine Fishes

Marine fishes face just the opposite problem from that of freshwater fishes. The salt content of sea water (~3%) is so hypertonic to that of their extracellular fluid that they are in continual danger of dehydration. The two major groups of marine fishes have solved this dilemma differently.

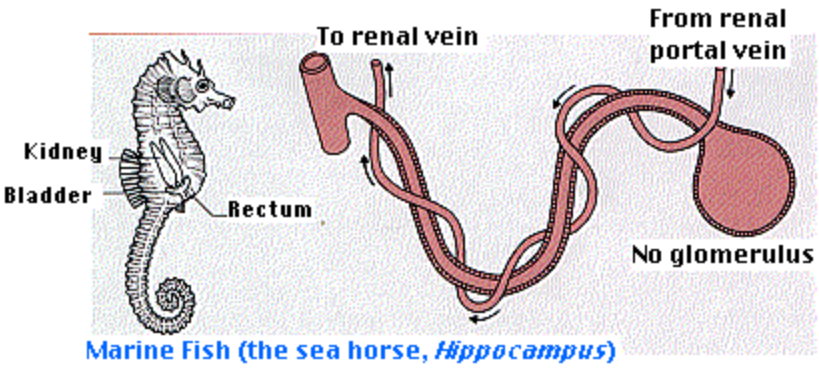


Figure 15.5.2.3 Marine kidneys

Cartilaginous Fishes (Chondrichthyes)

The cartilaginous fishes such as sharks, skates, and rays have developed high levels of urea in their blood. Shark's blood may contain 2.5% urea in contrast to the 0.01-0.03% in other vertebrates. This high level makes sharks blood isotonic to sea water, so the shark lives in osmotic balance with its environment and has a kidney that functions like ours with the exception that far more urea is reabsorbed in the shark's tubules than in ours.

Bony Fishes (Osteichthyes)

Marine bony fishes have solved the problem differently. They do lose water continuously but replace it by drinking sea water and then desalting it. The salt is returned to the sea by active transport at the gills. Living in constant danger of dehydration by the hypertonic sea, there is no reason to pump out large amounts of nephric filtrate at the glomerulus. The less water placed in the tubules, the less that has to be reabsorbed. So it is not surprising that many bony fishes have small glomeruli and some have no glomeruli at all. With a reduction in the filtration-reabsorption mechanism, the marine bony fishes rely more on tubular secretion for eliminating excess or waste solutes. Tubular secretion requires a good blood supply to the tubules. Lacking efficient glomeruli, the **renal portal** system must carry most of the burden.

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15.5C: Urea Cycle

Urea is the chief nitrogenous waste of mammals. Most of our nitrogenous waste comes from the breakdown of amino acids. This occurs by deamination.

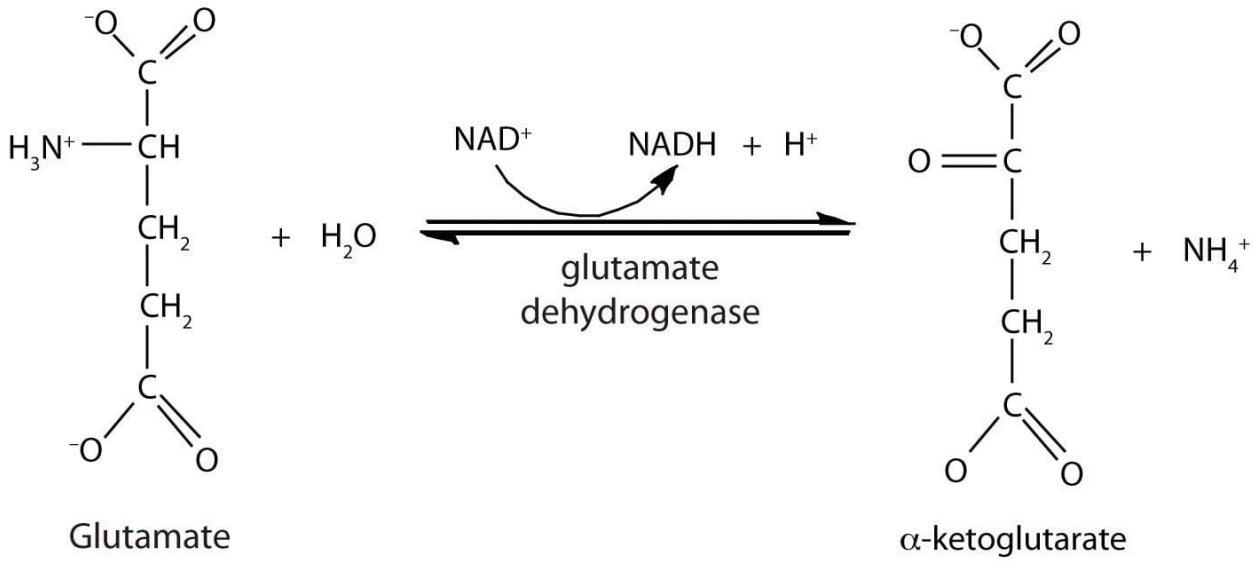


Figure 15.5.3.1 Deamination

Deamination of amino acids results in the production of **ammonia** (**NH3**). Ammonia is an extremely toxic base and its accumulation in the body would quickly be fatal. However, the **liver** contains a system of carrier molecules and enzymes which quickly converts the ammonia (and carbon dioxide) into urea. This is called the **urea cycle**.

The Urea Cycle

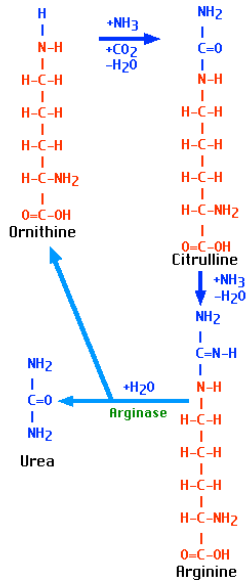


Figure 15.5.3.2 The Urea cycle

One turn of the cycle:

- consumes **2** molecules of **ammonia**

- consumes 1 molecule of carbon dioxide
- creates 1 molecule of **urea** ($(NH_2)_2CO$
- regenerates a molecule of **ornithine** for another turn.

Although our bodies cannot tolerate high concentrations of urea, it is much less poisonous than ammonia. Urea is removed efficiently by the kidneys.

Problems in Urea Cycle

There are several inherited diseases of the urea cycle caused by mutations in genes encoding one or another of the necessary enzymes. The most common of these is an inherited deficiency of **ornithine transcarbamylase**, an enzyme needed for the conversion of ornithine to citrulline. It results in elevated levels of ammonia that may be so high as to be life-threatening. It is an X-linked disorder; therefore most commonly seen in males. It can be **cured** by a liver transplant. It can also be **caused** by a liver transplant! In 1998, an Austrian woman was given a new liver from a male cadaver who - unknown to the surgeons - had a mutation in his single ornithine transcarbamylase gene. The woman's blood level of ammonia shot up, and she died a few days later.

Uric acid

Humans also excrete a second nitrogenous waste, **uric acid**. It is the product of **nucleic acid**, not protein, metabolism. It is produced within peroxisomes. Uric acid is only slightly soluble in water and easily precipitates out of solution forming needlelike crystals of sodium urate. These contribute to the formation of kidney stones and produce the excruciating pain of **gout** when deposited in the joints.

Curiously, our kidneys reclaim most of the uric acid filtered at the glomeruli. Why, if it can cause problems?

- Uric acid is a potent antioxidant and thus can protect cells from damage by reactive oxygen species (**ROS**).
- The concentration of uric acid is 100-times greater in the cytosol than in the extracellular fluid. So when lethally-damaged cells release their contents, crystals of uric acid form in the vicinity. These enhance the ability of nearby dendritic cells to "present" any antigens released at the same time to T cells leading to a stronger immune response.

So the risk of kidney stones and gout may be the price we pay for these protections.

Most mammals have an enzyme - **uricas** - for breaking uric acid down into a soluble product. However, during the evolution of great apes and humans, the gene encoding uricase became inactive. A predisposition to gout is our legacy.

Uric acid is the **chief** nitrogenous waste of insects, lizards and snakes and birds. It is the whitish material that birds leave on statues. These animals convert the waste products of **protein** metabolism as well as nucleic acid metabolism into uric acid. Because of its low solubility in water, these animals are able to eliminate waste nitrogen with little loss of water.

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15.6: HORMONES



- Topic hierarchy

15.6.1: Human Hormones

15.6.1.1: Thyroid and Parathyroids

15.6.1.2: Hormones of the Gut

15.6.1.3: Hormones of the Pancreas

15.6.1.4: Hormones of the Pituitary

15.6.1.5: Hormones of the Hypothalamus

15.6.1.6: Adrenal Glands

15.6.1.7: Sex Hormones

15.6.1.8: Progesterone

15.6.1.9: Melatonin and the Pineal Gland

15.6.1.10: Hormones of Kidney, Skin and Heart

15.6.1.11: Leptin - the Fat Hormone

15.6.1.12: Hormones of the Liver

15.6.1.13: Melanocyte Stimulating Hormone (MSH)

15.6.2: Insect Hormones

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15.6.1: Human Hormones

The essence of multicellularity is the coordinated interaction of the various kinds of cells that make up the body. Cells communicate with each other by chemical signals. Three kinds of chemical signaling can be distinguished:

- **autocrine**: the cell signals itself through a chemical that it synthesizes and then responds to. Autocrine signaling can occur solely within the cytoplasm of the cell or by a secreted chemical interacting with receptors on the surface of the same cell
- **paracrine**: chemical signals that diffuse into the area and interact with receptors on nearby cells. Examples include the release of cytokines that cause an inflammatory response in the area and the release of neurotransmitters at synapses in the nervous system.
- **endocrine**: the chemicals are secreted into the blood and carried by blood and tissue fluids to the cells they act upon.

This page will examine the properties of endocrine signaling.

Kinds of Hormones

There are two major classes of hormone: (1) proteins, peptides, and modified amino acids and (2) steroids.

Proteins, peptides and modified amino acids

These hydrophilic (and mostly large) hormone molecules bind to receptors on the surface of "target" cells; that is, cells able to respond to the presence of the hormone. These receptors are transmembrane proteins. Binding of the hormone to its receptor initiates a sequence of intracellular signals that may alter the behavior of the cell (such as by opening or closing membrane channels) or stimulate (or repress) gene expression in the nucleus by turning on (or off) the promoters and enhancers of the genes

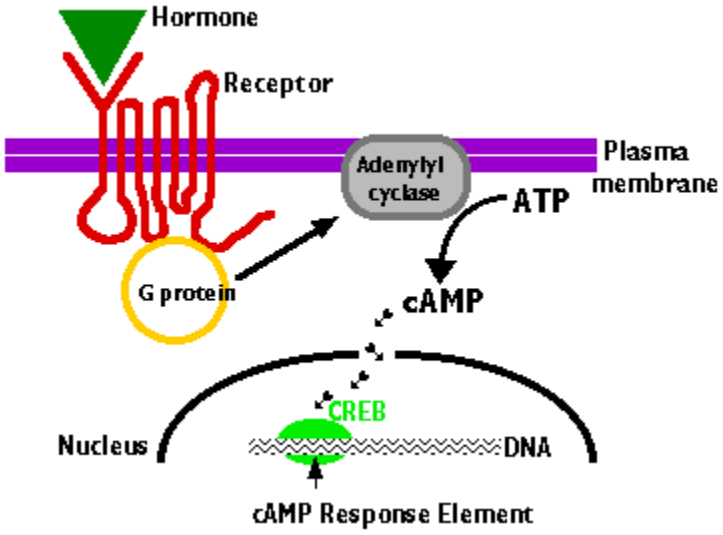


Figure 15.6.1.1: Protein hormone

This is the sequence of events:

- The hormone binds to a site on the extracellular portion of the receptor.
  - The receptors are transmembrane proteins that pass through the plasma membrane 7 times, with their N-terminal exposed at the exterior of the cell and their C-terminal projecting into the cytoplasm.
- Binding of the hormone to the receptor
  - activates a **G protein** associated with the cytoplasmic C-terminal
  - This initiates the production of a **"second messenger"**. The most common of these are
    - **cyclic AMP**, (**cAMP**) which is produced by **adenylyl cyclase** from **ATP**
    - **inositol 1,4,5-trisphosphate** (**IP3**)

- The second messenger, in turn, initiates a series of intracellular events (shown here as short arrows) such as
  - phosphorylation and activation of enzymes
  - release of Ca^{2+} into the cytosol from stores within the endoplasmic reticulum
- In the case of cAMP, these enzymatic changes activate the **transcription factor CREB** (**c**AMP **r**esponse **e**lement **b**inding protein).
- Once bound to its **response element** 5' TGACGTCA 3' in the promoters of genes that are able to respond to the hormone, activated CREB turns on gene transcription.
- The cell begins to produce the appropriate gene products in response to the hormonal signal it had received at its surface.

Steroid Hormones

Steroid hormones, being hydrophobic molecules, diffuse freely into all cells. However, their "target" cells contain cytoplasmic and/or nuclear proteins that serve as receptors of the hormone. The hormone binds to the receptor and the complex binds to **hormone response elements** — stretches of DNA within the promoters of genes responsive to the hormone. The hormone/receptor complex acts as a transcription factor turning target genes "on" (or "off").

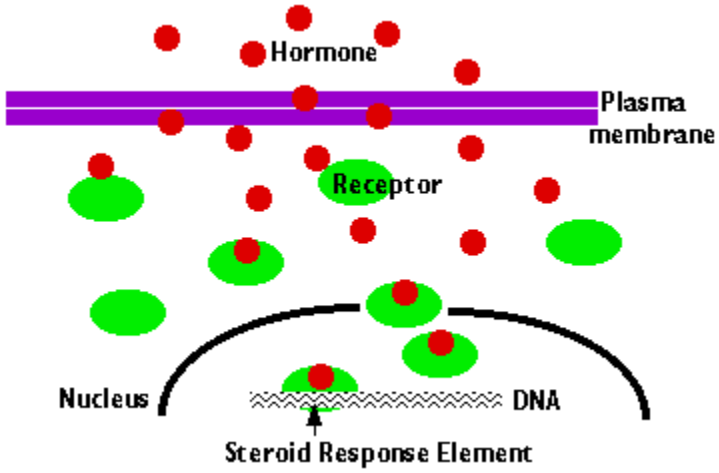


Figure 15.6.1.2 Steroid receptors

Hormone Regulation

The levels of hormones circulating in the blood are tightly controlled by three homeostatic mechanisms:

1. When one hormone stimulates the production of a second, the second suppresses the production of the first. Example: The follicle stimulating hormone (**FSH**) stimulates the release of estrogens from the ovarian follicle. A high level of estrogen, in turn, suppresses the further production of FSH.
2. Antagonistic pairs of hormones. Example: Insulin causes the level of blood sugar (glucose) to drop when it has risen. Glucagon causes it to rise when it has fallen.
3. Hormone secretion is increased (or decreased) by the same substance whose level is decreased (or increased) by the hormone. Example: a rising level of Ca^{2+} in the blood suppresses the production of the parathyroid hormone (PTH). A low level of Ca^{2+} stimulates it.

Hormone Transport

Although a few hormones circulate simply dissolved in the blood, most are carried in the blood bound to plasma proteins. For example, all the steroid hormones, being highly hydrophobic, are transported bound to plasma proteins.

Summary Table of Human Hormaones

Hormone	Structure (1)	Principal Source
Thyroid-stimulating hormone (TSH)	protein (201)	Anterior lobe of pituitary
Follicle-stimulating hormone (FSH)	protein (204)	
Luteinizing hormone (LH)	protein (204)	
Prolactin (PRL)	protein (198)	
Growth hormone (GH)	protein (191)	
Adrenocorticotropic hormone (ACTH)	peptide (39)	
Vasopressin	peptide (9)	Posterior lobe of pituitary
Oxytocin	peptide (9)	
Thyrotropin-releasing hormone (TRH)	peptide (3)	Hypothalamus
Gonadotropin-releasing hormone (GnRH)	peptide (10)	
Growth hormone-releasing hormone (GHRH)	peptides (40, 44)	
Corticotropin-releasing hormone (CRH)	peptide (41)	
Somatostatin	peptides (14, 28)	
Dopamine	tyrosine derivative	
Melatonin	tryptophan derivative	Pineal gland
Thyroxine (T4)	tyrosine derivative	Thyroid Gland
Calcitonin	peptide (32)	
Parathyroid hormone (PTH)	protein (84)	Parathyroid glands
	protein (251)	Bone
Osteocalcin	peptide (49)	
Erythropoietin (EPO)	protein (166)	
Glucocorticoids (e.g., cortisol)	steroids	Adrenal cortex
Mineralocorticoids (e.g., aldosterone)	steroids	
Androgens (e.g., testosterone)	steroids	
Adrenaline (epinephrine)	tyrosine derivative	Adrenal medulla
Noradrenaline (norepinephrine)	tyrosine derivative	
Estrogens (e.g., estradiol)	steroid	Ovarian follicle
Progesterone	steroid	Corpus luteum and placenta
Human chorionic gonadotropin (HCG)	protein (237)	Trophoblast and placenta
Androgens (e.g., testosterone)	steroid	Testes
Insulin	protein (51)	Pancreas (Islets of Langerhans)
Glucagon	peptide (29)	
Somatostatin	peptides (14, 28)	
Amylin	peptide (37)	
Erythropoietin (EPO)	protein (166)	Kidney
Calcitriol	steroid derivative	

Hormone	Structure (1)	Principal Source
Calciferol (vitamin D3)	steroid derivative	Skin
Atrial-natriuretic peptide (ANP)	peptides (28, 32)	Heart
Gastrin	peptides (e.g., 14)	Stomach and intestine
Secretin	peptide (27)	
Cholecystokinin (CCK)	peptides (e.g., 8)	
Fibroblast Growth Factor 19 (FGF19)	protein (216)	
Incretins	peptides (e.g., 31, 42)	
Somatostatin	peptides (14, 28)	
Neuropeptide Y	peptide (36)	
Ghrelin	peptide (28)	
PYY3-36	peptide (34)	
Serotonin	tryptophan derivative	
	protein (70)	Liver
Angiotensinogen	protein (485)	
Thrombopoietin	protein (332)	
Hepcidin	peptide (25)	
Betatrophin	protein (193)	
Leptin	protein (167)	Fat cells (adipocytes)
Retinol Binding Protein 4	protein (~180)	
Adiponectin	protein (117)	
Asprosin	protein (140)	

Note: Numbers within parentheses indicate the number of amino acids in the protein or peptide(s).

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15.6.1.1: Thyroid and Parathyroids

The Thyroid Gland

The thyroid gland is a double-lobed structure located in the neck. Embedded in its rear surface are the four parathyroid glands. The thyroid gland synthesizes and secretes: thyroxine (T4), and triiodothyronine (T3) and calcitonin.

T4 and T3

Both hormones are derivatives of the amino acid tyrosine with four atoms of iodine in **T4** , three in **T3**. The thyroid secretes mainly (80%) T4 , but when T4 enters target cells, one atom of iodine is removed from it converting it into T3. T3 is the more potent of the two hormones. It has many effects. Among the most prominent of these are:

- an increase in metabolic rate (seen by a rise in body temperature and the uptake of oxygen)
- an increase in the rate and strength of the heart beat

The thyroid cells responsible for the synthesis of T4 and T3 take up circulating iodine from the blood and attach them to tyrosine residues in the protein **thyroglobulin**. This action, as well as the synthesis of the hormones, is stimulated by the binding of thyroid stimulating hormone (TSH; also known as thyrotropin) to transmembrane receptors at the cell surface.

3

- **Cretinism**: hypothyroidism in infancy and childhood leads to stunted growth and intelligence. Can be corrected by giving thyroxine if started early enough.
- **Myxedema**: hypothyroidism in adults leads to lowered metabolic rate and vigor. Corrected by giving thyroxine.
- **Goiter**: enlargement of the thyroid gland. Can be caused by:
  - inadequate iodine in the diet with resulting low levels of T4 and T3
  - an autoimmune attack against the thyroglobulin in the thyroid gland (called **Hashimoto's thyroiditis**)

  Why should a **hypo**thyroid disease produce an enlarged gland? The activity of the thyroid is under negative feedback control:
  - The synthesis and release of thyrotropin releasing hormone (TRH) and TSH is normally inhibited as the levels of T4 and T3 rise in the blood.
  - When the iodine supply is inadequate, T4 and T3 levels fall.
  - This stimulates the hypothalamus and pituitary to **increased** TRH and TSH activity respectively. This stimulates the thyroid gland to enlarge.
- The symptoms of hypothyroidism can also result from **inherited mutations** in the genes encoding:
  - the **receptor for TSH** (present on the surface of thyroid cells)
  - the **receptor for T3** (present in the nucleus of almost all cells)

  The T3 receptor is a nuclear protein bound to the **thyroid response element** in the promoters of the many genes whose expression is influenced by thyroid hormones. When its ligand, T3, binds to it, it becomes a transcription factor turning on the transcription of many genes.

- **Graves´ disease:** Autoantibodies against the TSH receptor bind to the receptor mimicking the effect of TSH binding. Result: excessive production of thyroid hormones. Graves´ disease is an example of an autoimmune disease.
- **Osteoporosis:** High levels of thyroid hormones suppress the production of TSH through the negative-feedback mechanism mentioned above. The resulting low level of TSH causes an increase in the numbers of bone-reabsorbing osteoclasts resulting in osteoporosis.

Calcitonin

Calcitonin is a polypeptide of 32 amino acids. The thyroid cells in which it is synthesized have receptors that bind calcium ions (Ca^{2+}) circulating in the blood. A rise in its level, such as would occur with the absorption of calcium from a meal, stimulates the cells to release calcitonin. Calcitonin prevents a sharp rise in blood calcium by inhibiting the uptake of Ca^{2+} from the small intestine and inhibiting the Ca^{2+}-releasing activity of osteoclasts.

Because it slows the loss of Ca^{2+} from bones, calcitonin has been examined as a possible treatment for **osteoporosis**, a weakening of the bones that is a leading cause of hip and other bone fractures in the elderly. Being a polypeptide, calcitonin cannot be given by mouth (it would be digested), and giving by injection is not appealing. However, inhaling calcitonin appears to be an effective way to get therapeutic levels of the hormone into the blood. A synthetic version of calcitonin (trade name = Miacalcin) is now available as a nasal spray.

The Parathyroid Glands

The parathyroid glands are 4 tiny structures embedded in the rear surface of the thyroid gland. They secrete **parathyroid hormone** (**PTH**) a polypeptide of 84 amino acids. PTH increases the concentration of Ca^{2+} in the blood in three ways. PTH promotes

- release of Ca^{2+} from the huge reservoir in the bones. (99% of the calcium in the body is incorporated in our bones.)
- reabsorption of Ca^{2+} from the fluid in the tubules in the kidneys
- absorption of Ca^{2+} from the contents of the intestine (this action is mediated by **calcitriol**, the active form of **vitamin D**.)

PTH also regulates the level of phosphate in the blood. Secretion of PTH reduces the efficiency with which phosphate is reclaimed in the proximal tubules of the kidney causing a drop in the phosphate concentration of the blood.

Control of the Parathyroids

The cells of the parathyroid glands have surface G-protein-coupled receptors that bind Ca^{2+} (the same type of receptor is found on the calcitonin-secreting cells of the thyroid and on the calcium absorbing cells of the kidneys). Binding of Ca^{2+} to this receptor **depresses** the secretion of PTH and thus leads to a lowering of the concentration of Ca^{2+} in the blood. Two classes of inherited disorders involving mutant genes encoding the Ca^{2+} receptor occur:

- **loss-of-function mutations** with the mutant receptor always "off". Patients with these mutations have high levels of Ca^{2+} in their blood and excrete small amounts of Ca^{2+} in their urine. These mutations cause **hyper**parathyroidism.
- **gain-of-function mutations** with the mutant receptor always "on" (as though it had bound Ca^{2+}). People with these mutations have low levels of Ca^{2+} in their blood and excrete large amounts of Ca^{2+} in their urine. These mutations cause **hypo**parathyroidism.

Rare autoimmune disorders can mimic one or the other of these inherited disorders. In each case, autoantibodies bind to the receptors.

- If these **inhibit** the receptors, they cause **hyper**parathyroidism.
- If they **activate** the receptors (like those in Graves' disease), they cause **hypo**parathyroidism.

Diseases of the thyroid: Hyperparathyroidism

Tumors in the parathyroids elevate the level of PTH causing a rise in the level of blood Ca^{2+} at the expense of calcium stores in the bones. So much calcium may be withdrawn from the bones that they become brittle and break.

Until recently, treatment has been the removal of most — but not all — of the parathyroid tissue (i.e. the goal is the removal of 3 1/2 glands). Now clinical trials have begun on a drug (designated R-568) that mimics the action of calcium on the parathyroids, resulting in a drop in PTH and blood Ca^{2+} and sparing the calcium stores in the bone.

Diseases of the thyroid: Hypoparathyroidism

Causes:

- accidental removal of or damage to the parathyroids during neck surgery
- inherited mutations in the PTH gene
- inherited predisposition to an autoimmune attack against the parathyroids (and other glands)
- inherited defect in the embryonic development of the parathyroids (DiGeorge syndrome)

Treatment:

- give calcium supplements
- give calcitriol (1,25[OH]$_2$ vitamin D$_3$)
- give teriparatide (Forteo®), a synthetic (by recombinant DNA) version of PTH (containing only the 34 amino acids at the N-terminal).

For reasons that are not yet clear, this drug when given in daily injections (because it would be digested if taken by mouth), promotes strong bones and thus has been approved as a treatment for osteoporosis. While continuous high levels of PTH weaken bones by removing calcium from them, periodic injections of this drug strengthen bone by increasing the number and activity of osteoblasts.

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15.6.1.2: Hormones of the Gut

Over two dozen hormones have been identified in various parts of the gastrointestinal system. Most of them are peptides and many of them are also found in other tissues, especially the brain. Many act in a *paracrine* manner as well as being carried in the blood as true hormones. Their importance to health is uncertain as no known deficiency disorders have been found for any of them. We shall look at 8 of them here:

1. gastrin
2. somatostatin
3. secretin
4. cholecystokinin (CCK)
5. fibroblast growth factor 19 (FGF19)
6. incretins
7. ghrelin
8. neuropeptide Y (NPY)
9. peptide YY_{3-36} (PYY_{3-36})

The endocrine cells of the small intestine also secrete serotonin and substance P.

Gastrin

Gastrin is a mixture of several peptides, of which the most active contains 14 amino acids. It is secreted by cells in the stomach and duodenum. It stimulates the exocrine cells of the stomach to secrete **gastric juice**, a mixture of **hydrochloric acid** and the proteolytic enzyme **pepsin**.

Somatostatin

This mixture of peptides is secreted by cells in the gastric glands of the stomach and acts on the stomach (thus a paracrine effect) where it inhibits the release of gastrin and hydrochloric acid, the duodenum where it inhibits the release of *secretin* and *cholecystokinin* and the pancreas where it inhibits the release of glucagon. Taken together, all of these actions lead to a reduction in the rate at which nutrients are absorbed from the contents of the intestine. Somatostatin is also secreted by the hypothalamus and the pancreas.

Secretin

It is a polypeptide of 27 amino acids and is secreted by cells in the duodenum when they are exposed to the acidic contents of the emptying stomach. It stimulates the exocrine portion of the **pancreas** to secrete **bicarbonate** into the pancreatic fluid (thus neutralizing the acidity of the intestinal contents).

Cholecystokinin (CCK)

A mixture of peptides, of which an octapeptide (8 amino acids) is the most active. It is secreted by cells in the duodenum and jejunum when they are exposed to food. It acts on the **gall bladder** stimulating it to contract and force its contents of bile into the intestine and on the **pancreas** stimulating the release of pancreatic digestive enzymes into the pancreatic fluid. CCK also acts on vagal neurons leading back to the medulla oblongata which give a **satiety signal** (i.e., "that's enough food for now").

Fibroblast Growth Factor 19 (FGF19)

A protein of 216 amino acids and is secreted by cells in the lower portion of the small intestine (the *ileum*). Travels in the hepatic portal system to the liver where is stimulates the the synthesis of bile acids, uptake of glucose and its conversion into glycogen. It also travels to the gall bladder where it relaxes its smooth muscle wall allowing filling (in contrast to CCK which contracts the

gall bladder).

Incretins

The release of insulin from the pancreas is much greater when glucose is ingested with food rather than injected intravenously. It is due to the fact that the arrival of food in the duodenum stimulates the release of polypeptides called incretins. The two most important are:

- glucagon-like peptide-1 (GLP-1) the most active version of which has 29 amino acids
- glucose-dependent insulinotropic polypeptide (GIP) of 42 amino acids.

Their effects:

- enhancing the ability of glucose to stimulate insulin secretion by the pancreas
- stimulating the ability of the tissues (e.g., liver and muscle) to take up glucose from the blood
- slowing the emptying of the stomach
- suppressing glucagon secretion
- suppressing appetite thus reducing food intake.

All the actions prevent a sharp rise in blood glucose when consuming a sugar-rich meal. Exenatide (Byetta®) and liraglutide (Victoza®) are synthetic peptides that mimic the action of GLP-1 but the effects are longer-lasting. They are being used to treat patients with type 2 diabetes.

Ghrelin

Ghrelin is a lipopeptide consisting of 28 amino acids with a covalently attached 8-carbon fatty acid. Ghrelin is secreted by endocrine cells in the stomach, especially when one is hungry and acts on the hypothalamus to **stimulate** feeding. This action counteracts the inhibition of feeding by *leptin* and PYY3-36. Ghrelin increases the deposition of adipose tissue in mice and rats and does not seem to increase their appetite (ghrelin knockout animals don't eat any more food than normal animals).

Neuropeptide Y (NPY)

Neuropeptide Y contains 36 amino acids. It is a potent feeding **stimulant** and causes increased storage of ingested food as fat. Neuropeptide Y is also secreted by neurons in the hypothalamus where it blocks the transmission of pain signals to the brain and induces a calming effect in laboratory animals exposed to stressful situations. Velneperit is a drug that blocks the action of neuropeptide Y on its receptors. It is in clinical trials for the treatment of obesity.

PYY3-36

Peptide YY3-36 contains 34 amino acids, many of them in the same positions as those in neuropeptide Y. But the action of PYY3-36 is just the reverse of that of NPY, being a potent feeding **inhibitor**. It is released by cells in the intestine after meals. The amount secreted increases with the number of calories ingested and especially when these are derived from proteins rather than carbohydrates or fats. (This may explain the efficacy of the protein-rich, carbohydrate-poor Atkins diet.) PYY3-36 acts on the hypothalamus to suppress appetite, the pancreas to increase its exocrine secretion of digestive juices, and the gall bladder to stimulate the release of bile.

The appetite suppression mediated by PYY3-36 works more slowly than that of cholecystokinin and more rapidly than that of leptin. In a recent human study, volunteers given PYY3-36 were less hungry and ate less food over the next 12 hours than those who received saline. Neither group knew what they were getting, but one of the side-effects of injected PYY3-36 is a feeling of nausea and a bad taste to food which might account for these results!

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15.6.1.3: Hormones of the Pancreas

The bulk of the pancreas is an exocrine gland secreting pancreatic fluid into the duodenum after a meal. However, scattered through the pancreas are several hundred thousand clusters of cells called islets of Langerhans. The islets are endocrine tissue containing four types of cells. In order of abundance, they are the:

- beta cells, which secrete insulin and amylin
- alpha cells, which secrete glucagon
- delta cells, which secrete somatostatin
- gamma cells, which secrete pancreatic polypeptide

Beta Cells

Insulin is a small protein consisting of an alpha chain of 21 amino acids linked by two disulfide (S—S) bridges to a beta chain of 30 amino acids. Beta cells have channels in their plasma membrane that serve as glucose detectors. Beta cells secrete insulin in response to a rising level of circulating glucose ("blood sugar"). Insulin affects many organs. It stimulates skeletal muscle fibers totake up glucose and convert it into glycogen. Insulin also take up amino acids from the blood and convert them into protein. It

- acts on liver cells
  - stimulating them to take up glucose from the blood and convert it into glycogen while
  - inhibiting production of the enzymes involved in breaking glycogen back down ("glycogenolysis") and
  - inhibiting "gluconeogenesis"; that is, the conversion of fats and proteins into glucose.
- acts on fat (adipose) cells to stimulate the uptake of glucose and the synthesis of fat
- acts on cells in the hypothalamus to reduce appetite

In each case, insulin triggers these effects by binding to the insulin receptor - a transmembrane protein embedded in the plasma membrane of the responding cells. Taken together, all of these actions result in:

- the storage of the soluble nutrients absorbed from the intestine into insoluble, energy-rich products (glycogen, protein, fat)
- a drop in the level of blood sugar

Diabetes Mellitus

Diabetes mellitus is an endocrine disorder characterized by many signs and symptoms. Primary among these are a failure of the kidney to efficiently reclaim glucose so that glucose spills over into the urine and a resulting increase in the volume of urine because of the osmotic effect of this glucose (it reduces the return of water to the blood).

Diabetes mellitus is a disorder quite distinct from the similarly-named *diabetes insipidus*. They both result in the production of large amounts of urine (diabetes), but in one the urine is sweet while in the other (caused by ADH deficiency) it is not. Before the days of laboratory tests, a simple taste test ("mellitus" or "insipidus") enabled the doctor to make the correct diagnosis. There are three categories of diabetes mellitus:

- Type 1
- Type 2
- Inherited Forms of Diabetes Mellitus

Type 1 Diabetes Mellitus

(also known as Insulin-Dependent Diabetes Mellitus or IDDM)

- is characterized by little (hypo) or no circulating insulin
- most commonly appears in childhood
- it results from destruction of the beta cells of the islets
- the destruction results from a cell-mediated autoimmune attack against the beta cells

- What triggers this attack is still a mystery. One possibility: peptides derived from insulin may bond to unrelated peptides to form a "neoantigen"; that is, an antigen that was not present when tolerance to self-antigens was being established.

Type 1 diabetes is controlled by carefully-regulated injections of insulin. Insulin cannot be taken by mouth because, being a protein, it would be digested. However, the U.S. FDA has approved [in January 2006] an insulin inhaler that delivers insulin through the lungs and may reduce the number of daily injected doses needed.

Injections of insulin must be done carefully. Injections after vigorous exercise or long after a meal may drive the blood sugar level down to a dangerously low value causing an insulin reaction. The patient becomes irritable, fatigued, and may lose consciousness. If the patient is still conscious, giving a source of sugar (e.g., candy) by mouth usually solves the problem quickly. Injections of glucagon are sometimes used.

Type 2 Diabetes Mellitus

Type 2 is also known as Non Insulin-Dependent Diabetes Mellitus (NIDDM) and adult-onset diabetes. However, this type eventually leads to insulin dependence and also is now appearing in many children so those terms are no longer appropriate. Many people develop Type 2 diabetes mellitus without an accompanying drop in insulin levels (at least at first). In many cases, the problem appears to be a failure to express a sufficient number of glucose transporters in the plasma membrane (and T-system) of their skeletal muscles.

Normally when insulin binds to its receptor on the cell surface, it initiates a chain of events that leads to the insertion in the plasma membrane of increased numbers of a transmembrane glucose transporter (called GLUT4). This transporter forms a channel that permits the facilitated diffusion of glucose into the cell.

Skeletal muscle is the major "sink" for removing excess glucose from the blood (and converting it into glycogen). In type 2 diabetes, the patient's ability to remove glucose from the blood and convert it into glycogen may be only 20% of normal. This is called insulin resistance. Curiously, vigorous exercise seems to increase the expression of the glucose transporter on skeletal muscle and this may explain why type 2 diabetes is more common in people who live sedentary lives.

Type 2 diabetes mellitus usually strikes in adults and, particularly often, in overweight people. However, over the last few years in the U. S., the incidence of type 2 diabetes in children has grown to the point where they now account for 20% of all newly-diagnosed cases (and, like their adult counterparts, are usually overweight). Several drugs, all of which can be taken by mouth, are useful in restoring better control over blood sugar in patients with type 2 diabetes. However, late in the course of disease, patients may have to begin to take insulin. It is as though after years of pumping out insulin in an effort to overcome the patient's insulin resistance, the beta cells become exhausted.

Inherited Forms of Diabetes Mellitus

Some cases of diabetes result from mutant genes inherited from one or both parents. Examples:

- mutant genes for one or another of the transcription factors needed for transcription of the insulin gene (5 mutant versions have been identified).
- mutations in one or both copies of the gene encoding the insulin receptor. These patients usually have extra-high levels of circulating insulin but defective receptors. The mutant receptors
  - may fail to be expressed properly at the cell surface
  - may fail to transmit an effective signal to the interior of the cell.
- a mutant version of the gene encoding glucokinase, the enzyme that phosphorylates glucose in the first step of glycolysis.
- mutations in the gene encoding part of potassium channels in the plasma membrane of the beta cell. The channels fail to close properly causing the cell to become hyperpolarized and blocking insulin secretion.
- mutations in several mitochondrial genes which reduce insulin secretion by beta cells. These diseases are inherited from the mother as only her mitochondria survive in the fertilized egg.

While symptoms usually appear in childhood or adolescence, patients with inherited diabetes differ from most children with type 2 diabetes in having a history of diabetes in the family and not being obese.

insulin

For many years, insulin extracted from the glands of cows and pigs was used. However, pig insulin differs from human insulin by one amino acid; beef insulin by three. Although both work in humans to lower blood sugar, they are seen by the immune system as "foreign" and induce an antibody response in the patient that blunts their effect and requires higher doses. Two approaches have been taken to solve this problem:

- Convert pig insulin into human insulin by removing the one amino acid that distinguishes them and replacing it with the human version. This approach is expensive, so now the favored approach is to
- Insert the human gene for insulin into *E. coli* and grow recombinant human insulin in culture tanks. Insulin is not a glycoprotein so *E. coli* is able to manufacture a fully-functional molecule (trade name = Humulin). Yeast is also used (trade name = Novolin).

Recombinant DNA technology has also made it possible to manufacture slightly-modified forms of human insulin that work faster (Humalog® and NovoLog®) or slower (Lantus®) than regular human insulin.

Amylin

Amylin is a peptide of 37 amino acids, which is also secreted by the beta cells of the pancreas. Some of its actions include inhibits the secretion of glucagon, slows the emptying of the stomach, sends a satiety signal to the brain. All of its actions tend to supplement those of insulin, reducing the level of glucose in the blood. A synthetic, modified, form of amylin (pramlintide or Symlin®) is used in the treatment of type 2 diabetes.

Alpha Cells

The alpha cells of the islets secrete glucagon, a polypeptide of 29 amino acids. Glucagon acts principally on the liver where it stimulates the conversion of glycogen into glucose ("glycogenolysis") and fat and protein into intermediate metabolites that are ultimately converted into glucose ("gluconeogenesis"). In both cases, the glucose is deposited in the blood.



X-Ray Crystal Structure of Glucagon based on PDB 1GCN. (CC BY-SA 3.0; Truthortruth).

Glucagon secretion is

- stimulated by low levels of glucose in the blood;
- inhibited by high levels, and
- inhibited by amylin.

The physiological significance of this is that glucagon functions to maintain a steady level of blood sugar level between meals. Injections of glucagon (which is readily available thanks to recombinant DNA technology) are sometimes given to diabetics suffering from an insulin reaction in order to speed the return of normal levels of blood sugar.

Delta Cells

The delta cells secrete *somatostatin*, which consists of two polypeptides, one of 14 amino acids and one of 28. Somatostatin has a variety of functions. Taken together, they work to reduce the rate at which food is absorbed from the contents of the intestine. Somatostatin is also secreted by the hypothalamus and by the intestine.

Gamma Cells

The gamma cells of the islets secrete a 36-amino-acid pancreatic polypeptide, which reduces appetite.

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15.6.1.4: Hormones of the Pituitary

The pituitary gland is pea-sized structure located at the base of the brain. In humans, it consists of two lobes: the Anterior Lobe and the Posterior Lobe.

Hormones of the Anterior Lobe

The anterior lobe contains six types of secretory cells, all but one of which are specialized to secrete only one of the anterior lobe hormones. All of them secrete their hormone in response to hormones reaching them from the hypothalamus of the brain.

Thyroid Stimulating Hormone (TSH)

TSH (also known as thyrotropin) is a glycoprotein consisting of a beta chain of 118 amino acids and an alpha chain of 92 amino acids. The alpha chain is identical to that found in two other pituitary hormones, FSH and LH as well as in the hormone chorionic gonadotropin. Thus it is its beta chain that gives TSH its unique properties. The secretion of TSH is stimulated by the arrival of thyrotropin releasing hormone (TRH) from the hypothalamus and is inhibited by the arrival of somatostatin from the hypothalamus.

As its name suggests, TSH stimulates the thyroid gland to secrete its hormone thyroxine (T_4). It does this by binding to transmembrane G-protein-coupled receptors (GPCRs) on the surface of the cells of the thyroid. Some people develop antibodies against their own TSH receptors. When these bind the receptors, they "fool" the cell into making more T_4 causing hyperthyroidism. The condition is called *thyrotoxicosis* or *Graves' disease*.

Hormone deficiencies

A deficiency of TSH causes hypothyroidism: inadequate levels of T_4 (and thus of T_3). Physicians occasionally encounter patients who are homozygous for mutant TSH receptors or mutant TRH receptors. In either case, they suffer from hypothyroidism. A deficiency of TSH, or mutant TSH receptors, have also been implicated as a cause of osteoporosis. Mice, whose TSH receptors have been knocked out, develop increased numbers of bone-reabsorbing osteoclasts.

Follicle-Stimulating Hormone (FSH)

FSH is a heterodimeric glycoprotein consisting of the same alpha chain found in TSH (and LH) and a beta chain of 118 amino acids, which gives it its unique properties. Synthesis and release of FSH is triggered by the arrival from the hypothalamus of gonadotropin-releasing hormone (GnRH). The effect of FSH depends on one's sex. In sexually-mature females, FSH (assisted by LH) acts on the follicle to stimulate it to release estrogens. FSH produced by recombinant DNA technology (Gonal-f®) is available to promote ovulation in women planning to undergo *in vitro* fertilization (IVF) and other forms of assisted reproductive technology. In sexually-mature males, FSH acts on spermatogonia stimulating (with the aid of testosterone) the production of sperm.

Luteinizing Hormone (LH)

LH is synthesized within the same pituitary cells as FSH and under the same stimulus (GnRH). It is also a heterodimeric glycoprotein consisting of the same 92-amino acid alpha subunit found in FSH and TSH (as well as in chorionic gonadotropin) and a beta chain of 121 amino acids that is responsible for its properties.

The effects of LH also depend on sex. In sexually-mature females, a surge of LH triggers the completion of meiosis I of the egg and its release (ovulation) in the middle of the menstrual cycle; LH also stimulates the now-empty follicle to develop into the corpus luteum, which secretes progesterone during the latter half of the menstrual cycle. Women with a severe LH deficiency can now be treated with human LH (Luveris®) produced by recombinant DNA technology. LH in males acts on the interstitial cells (also known as Leydig cells) of the testes stimulating them to synthesize and secrete the male sex hormone, testosterone. LH in males is also known as interstitial cell stimulating hormone (ICSH).

Prolactin (PRL)

Prolactin is a protein of 198 amino acids. During pregnancy it helps in the preparation of the breasts for future milk production. After birth, prolactin promotes the synthesis of milk. Prolactin secretion is stimulated by TRH and repressed by estrogens and dopamine. In pregnant mice, prolactin stimulates the growth of new neurons in the olfactory center of the brain.

Growth Hormone (GH)

Human growth hormone (HGH; also called somatotropin) is a protein of 191 amino acids. The GH-secreting cells are stimulated to synthesize and release GH by the intermittent arrival of growth hormone releasing hormone (GHRH) from the hypothalamus. GH promotes body growth by:

- binding to receptors on the surface of liver cells.
- This stimulates them to release insulin-like growth factor-1 (IGF-1; also known as somatomedin)
- IGF-1 acts directly on the ends of the long bones promoting their growth

Things that can go wrong:

- In childhood,
  - hyposecretion of GH produces a short but normally-proportioned body.
  - Growth retardation can also result from an inability to respond to GH. This can be caused by inheriting two mutant genes encoding the receptors for
    - GHRH or
    - GH (causing Laron syndrome, a form of dwarfism) or
    - homozygosity for a disabling mutation in *STAT5b*, which is part of the "downstream" signaling process after GH binds its receptor.
  - hypersecretion leads to gigantism
- In adults, a hypersecretion of GH or GHRH leads to *acromegaly*.

Hormone-replacement therapy

GH from domestic mammals like cows and pigs does not work in humans. So for many years, the only source of GH for therapy was that extracted from the glands of human cadavers. But this supply was shut off when several patients died from a rare neurological disease attributed to contaminated glands. Now, thanks to recombinant DNA technology, recombinant human GH (rHGH) is available. While a benefit to patients suffering from GH deficiency or the short stature associated with Turner syndrome, there has also been pressure to use it to stimulate growth in youngsters who have no deficiency but whose parents want them to grow up tall. So, in the summer of 2003, the U.S. FDA approved the use of human growth hormone (HGH) for boys predicted to grow no taller than 5′3″ and for girls, 4′11″ even though otherwise perfectly healthy.

ACTH — the adrenocorticotropic hormone

ACTH is a peptide of 39 amino acids. It is cut from a larger precursor proopiomelanocortin (POMC). ACTH acts on the cells of the adrenal cortex, stimulating them to produce

- glucocorticoids, like cortisol
- mineralocorticoids, like aldosterone
- androgens (male sex hormones, like testosterone)
- In the fetus, ACTH stimulates the adrenal cortex to synthesize a precursor of estrogen called dehydroepiandrosterone sulfate (DHEA-S) which helps prepare the mother for giving birth.

Production of ACTH depends on the intermittent arrival of corticotropin-releasing hormone (CRH) from the hypothalamus. Hypersecretion of ACTH is a frequent cause of Cushing's syndrome.

Alpha Melanocyte-Stimulating Hormone (α-MSH)

Alpha MSH is also a cleavage product of proopiomelanocortin (POMC). In fact, α-MSH is identical to the first 13 amino acids at the amino terminal of ACTH. MSH is discussed in a separate page.

Hormones of the Posterior Lobe

The posterior lobe of the pituitary releases two hormones — both synthesized in the hypothalamus — *vasopressin* and *oxytocin* into the circulation.

Vasopressin

Vasopressin is a peptide of 9 amino acids (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly). It is also known as arginine vasopressin (AVP) and the antidiuretic hormone (ADH). Vasopressin acts on the collecting ducts of the kidney to facilitate the reabsorption of water into the blood. Thus it acts to reduce the volume of urine formed (giving it its name of antidiuretic hormone). A deficiency of vasopressin or inheritance of mutant genes for its receptor (called V2) leads to excessive loss of urine, a condition known as diabetes insipidus. The most severely-afflicted patients may urinate as much as 30 liters (almost 8 gallons!) of urine each day. The disease is accompanied by terrible thirst, and patients must continually drink water to avoid dangerous dehydration.

Another type of receptor for vasopressin (designated V1a) is found in the brain, e.g., in voles and mice (rodents) and in primates like monkeys and humans.

- Male prairie voles (*Microtus pinetorum*) and marmoset monkeys have high levels of the V1a receptor in their brains, tend to be monogamous, and help with care of their young.
- Male meadow voles (*Microtus montanus*) and rhesus monkeys have lower levels of the V1a receptor in their brains, are promiscuous, and give little or no help with the care of their young.

Meadow voles whose brains have been injected with a vector causing increased expression of the V1a receptor become more like prairie voles in their behavior. (See Lim, M. M. *et al*., Nature, 17 June 2004.)

The level of expression of the V1a receptor gene is controlled by a "microsatellite" region upstream (5') of the ORF. This region contains from 178 to 190 copies of a repeated tetranucleotide (e.g., CAGA). Prairie voles have more copies of the repeat than meadow voles, and they express higher levels of the receptor in the parts of the brain associated with these behaviors. A similar microsatellite region is present in the pygmy chimpanzee or bonobo (*Pan paniscus*) but is much shorter in the less-affectionate common chimpanzee (*Pan troglodytes*).

Vasopressin and the Circadian Clock

Mice are nocturnal and become active at the start of the night. This is a circadian rhythm that persists for a time even after the lights in the lab are turned off each day 8 hours sooner (like arriving in London after a flight from Los Angeles, California). Only after 8–10 days do the mice overcome their "jet lag", adjusting to the new dark-light schedule. (It also takes us about one day to reset our circadian rhythms for each hour that our day-night schedule is shifted.)

It turns out that arginine vasopressin, acting on the suprachiasmatic nucleus (SCN), plays a role in this resistance to resetting their circadian clock. Mice with their genes for the V1a and V1b receptors knocked out adjust much more quickly (2–4 days) to the change. What evolutionary advantage this resistance to resetting the circadian clock confers is not clear, but understanding the mechanism raises the possibility of using drugs to speed getting over jet lag and also to help those whose work shifts are periodically altered. (Read about this work in Yamaguchi, Y., *et al*. in the 4 October 2013 issue of Science.).

Oxytocin

Oxytocin is a peptide of 9 amino acids (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly). It acts on certain smooth muscles by stimulating contractions of the uterus at the time of birth and stimulating release of milk when the baby begins to suckle. Oxytocin is often given to prospective mothers to hasten birth.

In rodents, oxytocin also acts on the nucleus *accumbens* and *amygdala* in the brain where it enhances bonding between males and females after they have mated and bonding between a mother and her newborn. In mice, oxytocin acts on striated muscle stem cells to promote repair after they have been injured. In humans, oxytocin increases the level of one's trust in other people.

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15.6.1.5: Hormones of the Hypothalamus

The hypothalamus is a region of the brain. It contains several types of neurons responsible for secreting different hormones.

- Thyrotropin-releasing hormone (TRH)
- Gonadotropin-releasing hormone (GnRH)
- Growth hormone-releasing hormone (GHRH)
- Corticotropin-releasing hormone (CRH)
- Somatostatin
- Dopamine

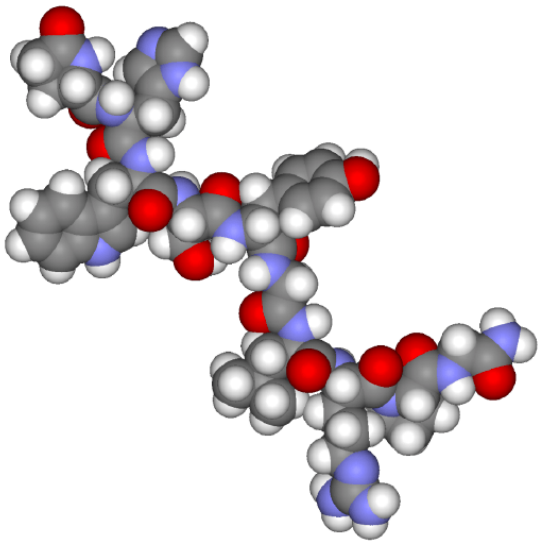
All of these are released into the blood in the capillaries and travel immediately – in portal veins – to a second capillary bed in the anterior lobe of the pituitary, where they exert their effects. All of them are released in periodic spurts. In fact, replacement hormone therapy with these hormones does not work unless the replacements are also given in spurts. Two other hypothalamic hormones *vasopressin* and *oxytocin* travel in the neurons themselves to the posterior lobe of the pituitary where they are released into the circulation.

Thyrotropin-releasing hormone (TRH)

TRH is a tripeptide (GluHisPro). When it reaches the anterior lobe of the pituitary it stimulates the release there of **thyroid-stimulating hormone** (**TSH**) and **prolactin** (**PRL**)

Gonadotropin-releasing hormone (GnRH)

GnRH is a peptide of 10 amino acids. Its secretion at the onset of puberty triggers sexual development, and from then on it is essential for normal sexual physiology in both males and females. In both sexes, its secretion occurs in periodic pulses usually occurring every 1–2 hours.



Space-filling model of gonadotropin-releasing hormone. (public domain, Fvasconcellos).

Primary Effects	Secondary Effects
FSH and LH **Up**	estrogen and progesterone **Up** (in females)
	testosterone **Up** (in males)

After puberty, a hyposecretion of GnRH may result from intense physical training or anorexia nervosa. Synthetic agonists of GnRH are used to treat inherited or acquired deficiencies of GnRH secretion and prostate cancer. In this case, continuous high levels of the GnRH agonist

- reduces the number of GnRH receptors in the pituitary, which
- reduces its secretion of FSH and LH, which
- reduces the secretion of testosterone, which
- reduces the stimulation of the cells of the prostate.

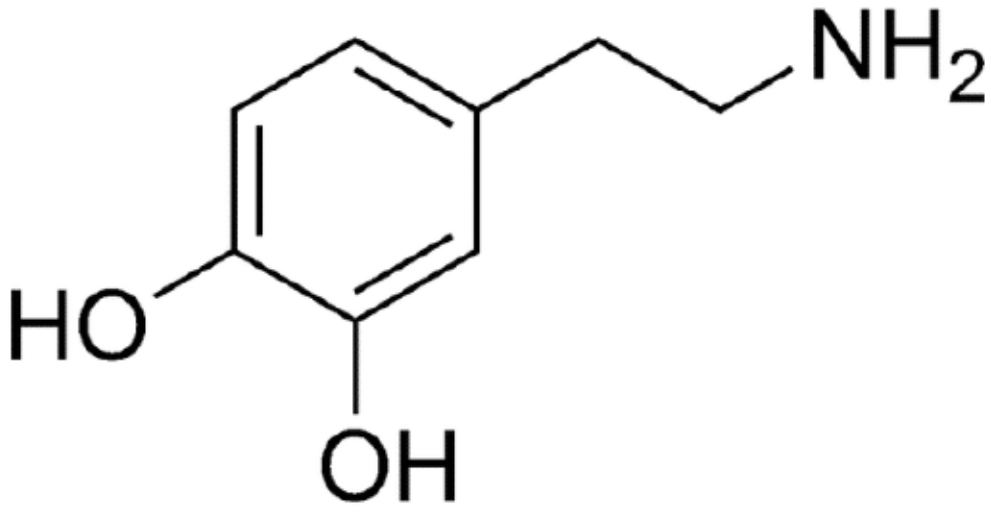
Growth hormone-releasing hormone (GHRH): GHRH is a mixture of two peptides, one containing 40 amino acids, the other 44. As its name indicates, GHRH stimulates cells in the anterior lobe of the pituitary to secrete growth hormone (GH).

Corticotropin-releasing hormone (CRH): CRH is a peptide of 41 amino acids. As its name indicates, its acts on cells in the anterior lobe of the pituitary to release adrenocorticotropic hormone (ACTH). CRH is also synthesized by the placenta and seems to determine the duration of pregnancy. It may also play a role in keeping the T cells of the mother from mounting an immune attack against the fetus.

Somatostatin: Somatostatin is a mixture of two peptides, one of 14 amino acids, the other of 28. Somatostatin acts on the anterior lobe of the pituitary to **inhibit** the release of growth hormone (GH) and **inhibit** the release of thyroid-stimulating hormone (TSH). Somatostatin is also secreted by cells in the pancreas and in the intestine where it inhibits the secretion of a variety of other hormones.

Dopamine

Dopamine is a derivative of the amino acid tyrosine. It mediates several functions in the brain, including **inhibiting** the release of prolactin (PRL) from the anterior lobe of the pituitary, modulating motor-control centers (a loss of dopamine-secreting cells produces Parkinson's disease), and activating the reward centers of the brain. Dopamine-secreting cells are also found in other parts of the body where most of its actions are paracrine; that is, acting on nearby cells.



Vasopressin and Oxytocin

These peptides are released from the posterior lobe of the pituitary and are described in the page devoted to the pituitary.

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15.6.1.6: Adrenal Glands

This page discusses the structure and function of the adrenal glands, detailing the outer adrenal cortex and inner medulla. The cortex produces key steroid hormones such as glucocorticoids and mineralocorticoids, which manage blood sugar and blood pressure, respectively. The medulla secretes adrenaline and noradrenaline during stress responses. It also mentions disorders like Addison's disease, associated with hormone deficiency, and Cushing's syndrome, caused by excess glucocorticoids.

The adrenal glands are two small structures situated one atop each kidney. Both in anatomy and in function, they consist of two distinct regions an outer layer, the **drenal ortex**, which surrounds the **adrenal medulla**.

The Adrenal Cortex

Using cholesterol as the starting material, the cells of the adrenal cortex secrete a variety of **steroid** hormones. These fall into three classes:

- glucocorticoids (e.g., cortisol)
- **mineralocorticoids** (e.g., aldosterone)
- **androgens** (e.g., testosterone)

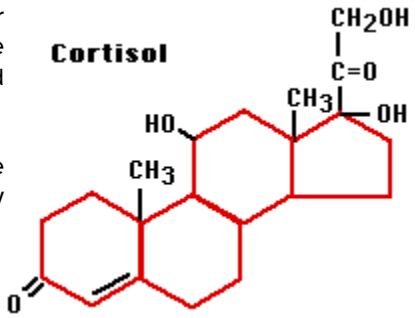
Production of all three classes is triggered by the secretion of ACTH from the anterior lobe of the pituitary.

These hormones achieve their effects by:

- Travelling through the body in the blood. Because they are so hydrophobic, they must be carried bound to a serum globulin.
- Entering from the blood into all cells.
- Binding to their **recepto -** a protein present in the cytoplasm and/or nucleus of "target" cells.
- The hormone-receptor complex binds to a second to form a **homodimer**.
- The homodimer migrates into the nucleus (if it did not form there) where it binds to specific **hormone response elements** in DNA.
- These are specific DNA sequences in the **promoter** of genes that will be turned on (or off) by the interaction.
- Other **transcription factors** are recruited to the promoter and gene transcription begins at some genes and is inhibited at others.

Glucocorticoids

The glucocorticoids get their name from their effect of raising the level of blood sugar (glucose). One way they do this is by stimulating **gluconeogenesis** in the liver: the conversion of fat and protein into intermediate metabolites that are ultimately converted into glucose.



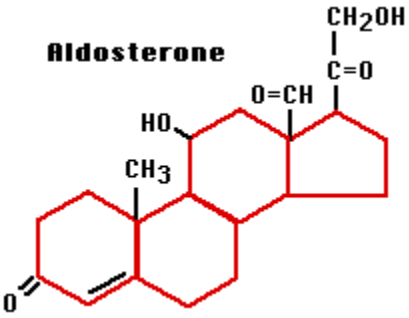
The most abundant glucocorticoid is **cortisol** (also called hydrocortisone). Cortisol and the other glucocorticoids also have a potent anti-inflammatory effect on the body. They depress the immune response, especially cell-mediated immune responses.

For this reason glucocorticoids are widely used in therapy:

- to reduce the inflammatory destruction of rheumatoid arthritis and other autoimmune diseases
- to prevent the rejection of transplanted organs
- to control asthma

Mineralocorticoids

The mineralocorticoids get their name from their effect on mineral metabolism. The most important of them is the steroid **aldosterone**. Aldosterone acts on the kidney promoting the reabsorption of sodium ions (Na^+) into the blood. Water follows the salt and this helps maintain normal blood pressure.



Aldosterone also

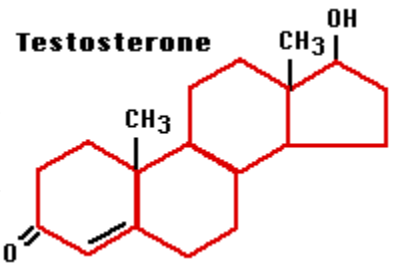
- acts on sweat glands to reduce the loss of sodium in perspiration
- acts on taste cells to increase the sensitivity of the taste buds to sources of sodium.

The secretion of aldosterone is stimulated by:

- a drop in the level of sodium ions in the blood
- a rise in the level of potassium ions in the blood
- angiotensin II
- ACTH (as is that of cortisol)

Androgens

The adrenal cortex secretes precursors to androgens such as **testosterone**.



In sexually-mature males, this source is so much lower than that of the testes that it is probably of little physiological significance. However, excessive production of adrenal androgens can cause premature puberty in young boys.

In females, the adrenal cortex is a major source of androgens. Their hypersecretion may produce a masculine pattern of body hair and cessation of menstruation.

Addison's Disease: Hyposecretion of the adrenal cortices

Addison's disease has many causes, such as

- destruction of the adrenal glands by infection
- their destruction by an autoimmune attack
- an inherited mutation in the ACTH receptor on adrenal cells

The essential role of the adrenal hormones means that a deficiency can be life-threatening. Fortunately, replacement therapy with glucocorticoids and mineralocorticoids can permit a normal life.

Cushing's Syndrome: Excessive levels of glucocorticoids

In Cushing's syndrome, the level of glucocorticoids, especially **cortisol**, is too high.

It can be caused by:

- excessive production of ACTH by the anterior lobe of the pituitary
- excessive production by the adrenals themselves (e.g., because of a tumor), or (quite commonly)
- as a result of glucocorticoid **therapy for some other disorder** such as rheumatoid arthritis or preventing the rejection of an organ transplant

The Adrenal Medulla

The adrenal medulla consists of masses of neurons that are part of the sympathetic branch of the autonomic nervous system.

Instead of releasing their neurotransmitters at a synapse, these neurons release them into the blood. Thus, although part of the nervous system, the adrenal medulla functions as an endocrine gland.

The adrenal medulla releases **adrenaline** (also called epinephrine) and **noradrenaline** (also called norepinephrine). Both are derived from the amino acid tyrosine. Release of adrenaline and noradrenaline is triggered by nervous stimulation in response to physical or mental stress. The hormones bind to **adrenergic receptors** — transmembrane proteins in the plasma membrane of many cell types.

Some of the effects are:

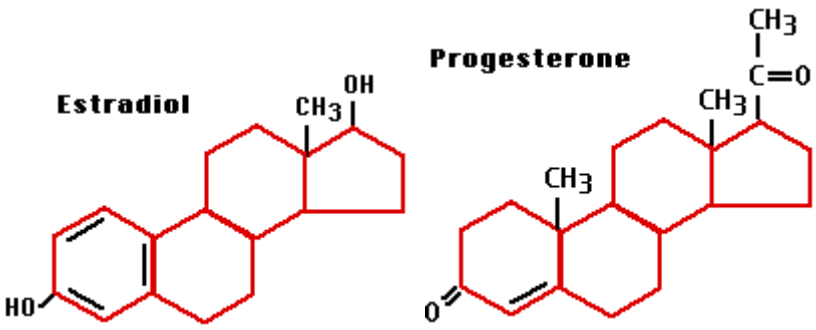
- increase in the rate and strength of the heartbeat resulting in increased blood pressure
- blood shunted from the skin and viscera to the skeletal muscles, coronary arteries, liver, and brain
- rise in blood sugar
- increased metabolic rate
- bronchi dilate
- pupils dilate
- hair stands on end ("gooseflesh" in humans)
- clotting time of the blood is reduced
- increased ACTH secretion from the anterior lobe of the pituitary

All of these effects prepare the body to take immediate and vigorous action.

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15.6.1.7: Sex Hormones

The ovaries of sexually-mature females secrete both a mixture of *estrogens (*of which 17 β-estradiol is the most abundant and most potent) and *progesterone*.



Estrogens

Estrogens are *steroids* and are primarily responsible for the conversion of girls into sexually-mature women including:

- development of breasts
- further development of the uterus and vagina
- broadening of the pelvis
- growth of pubic and axillary hair
- increase in adipose (fat) tissue
- participate in the monthly preparation of the body for a possible pregnancy
- participate in pregnancy if it occurs

Estrogens also have non-reproductive effects. For example, they antagonize the effects of the *parathyroid hormone*, minimizing the loss of calcium from bones and thus helping to keep bones strong. They also promote blood clotting.

Progesterone

Progesterone is also a steroid. It has many effects in the body, some having nothing to do with sex and reproduction. Here we shall focus on the role of progesterone in the menstrual cycle and pregnancy.

How estrogens and progesterone achieve their effects

Steroids like estrogens and progesterone are small, hydrophobic molecules that are transported in the blood bound to a serum globulin.

- In "target" cells, i.e., cells that change their gene expression in response to the hormone, they bind to receptor proteins located in the cytoplasm and/or nucleus.
- The hormone-receptor complex enters the nucleus (if it formed in the cytoplasm) and binds to specific sequences of DNA, called the estrogen (or progesterone) **response elements.**
- Response elements are located in the promoters of genes.
- The hormone-receptor complex acts as a transcription factor (often recruiting other transcription factors to help) which turns on (sometimes off) transcription of those genes.
- Gene expression in the cell produces the response.

Some "target" cells also have other types of estrogen and progesterone receptors that are embedded in a membrane (endoplasmic reticulum and plasma membrane respectively). Binding of the hormone to them produces more rapid effects than those of the nuclear receptors. For example, human sperm have receptors that within a second of being exposed to progesterone

activate the sperm to increased motility.

Regulation of Estrogen and Progesterone

The synthesis and secretion of estrogens is stimulated by **follicle-stimulating hormone** (FSH), which is, in turn, controlled by the **hypothalamic gonadotropin releasing hormone** (GnRH).

Hypothalamus	→	GnRH	→	Pituitary	→	FSH	→	Follicle	→	Estrogens

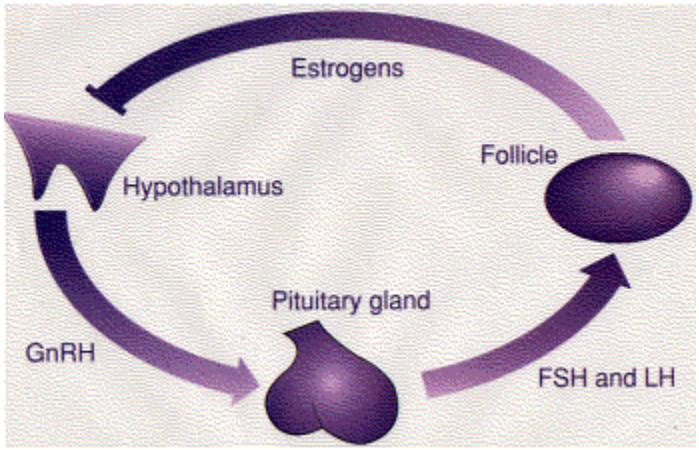


Figure 15.6.1.7.1 Hypothalamic feedback

High levels of estrogens **suppress** the release of GnRH (bar) providing a negative-feedback control of hormone levels.

It works like this: Secretion of GnRH depends on certain neurons in the hypothalamus which express a gene (*KISS-1*) encoding a protein of 145 amino acids. From this are cut several short peptides collectively called **kisspeptin**. These are secreted and bind to G-protein-coupled receptors on the surface of the GnRH neurons stimulating them to release GnRH. However, high levels of estrogen (or progesterone or testosterone) inhibit the secretion of kisspeptin and suppress further production of those hormones.

Progesterone production is stimulated by luteinizing hormone (LH), which is also stimulated by GnRH.

Hypothalamus	→	GnRH	→	Pituitary	→	LH	→	Corpus luteum	→	Progesterone

Elevated levels of progesterone control themselves by the same negative feedback loop used by estrogen (and testosterone).

The Menstrual Cycle

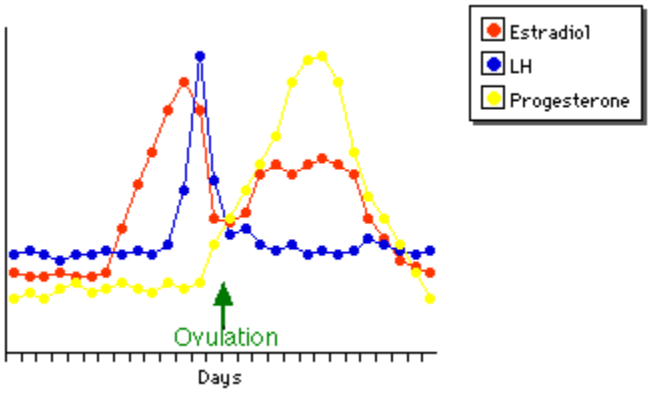


Figure 15.6.1.7.2 Menstrual cycle

About every 28 days, some blood and other products of the disintegration of the inner lining of the uterus (the *endometrium*) are discharged from the uterus, a process called **menstruation**. During this time a new follicle begins to develop in one of the ovaries. After menstruation ceases, the follicle continues to develop, secreting an increasing amount of **estrogen** as it does so.

- The rising level of estrogen causes the endometrium to become thicker and more richly supplied with blood vessels and glands.
- A rising level of **LH** causes the developing egg within the follicle to complete the first meiotic division (meiosis I), forming a **secondary oocyte**.
- After about two weeks, there is a sudden surge in the production of LH.
- This surge in LH triggers **ovulation**: the release of the secondary oocyte into the fallopian tube.
- Under the continued influence of LH, the now-empty follicle develops into a **corpus luteum** (hence the name luteinizing hormone for LH).
- Stimulated by LH, the corpus luteum secretes **progesterone** which
  - continues the preparation of the endometrium for a possible pregnancy
  - inhibits the contraction of the uterus
  - inhibits the development of a new follicle
- If fertilization does **not** occur (which is usually the case)
  - the rising level of progesterone inhibits the release of GnRH which, in turn, inhibits further production of progesterone
- As the progesterone level drops
  - the corpus luteum begins to degenerate
  - the endometrium begins to break down, its cells committing programmed cell death
  - the inhibition of uterine contraction is lifted
  - the bleeding and cramps of menstruation begin.

Fertilization

Fertilization of the egg is also influenced by progesterone. Sperm swim towards the egg by chemotaxis following a gradient of progesterone secreted by cells surrounding the egg. Progesterone opens CatSper ("cation sperm") channels in the plasma membrane surrounding the anterior portion of the sperm tail. This allows an influx of Ca^{2+} ions which causes the flagellum to beat more rapidly and vigorously.

Pregnancy

As the fertilized egg passes down the fallopian tube, it undergoes its first mitotic divisions. By the end of the week, the developing embryo has become a hollow ball of cells called a **blastocyst**. At this time, the blastocyst reaches the uterus and embeds itself in the endometrium, a process called **implantation**. With implantation, pregnancy is established.

The blastocyst has two parts:

- the **inner cell mass**, which will become the baby
- the **trophoblast**, which will
  - develop into the **placenta** and umbilical cord
  - begin to secrete **human chorionic gonadotropin** (**HCG**).

HCG is a glycoprotein. It is a heterodimer of the same alpha subunit (of 92 amino acids) used by TSH, FSH, and LH and a unique beta subunit (of 145 amino acids). HCG behaves much like FSH and LH with one crucial exception: it is **NOT** inhibited by a rising level of progesterone. Thus HCG prevents the deterioration of the corpus luteum at the end of the fourth week and enables pregnancy to continue beyond the end of the normal menstrual cycle. Because only the implanted trophoblast makes HCG, its early appearance in the urine of pregnant women provides the basis for the most widely used test for pregnancy (which can provide a positive signal even before menstruation would have otherwise begun). As pregnancy continues, the placenta becomes a major source of **progesterone**, and its presence is essential to maintain pregnancy. Mothers at risk of giving birth too soon can be given a synthetic progestin to help them retain the fetus until it is full-term.

Birth

Toward the end of pregnancy,

- The **placenta** releases large amounts of CRH which stimulates the **pituitary glands** of both mother and her fetus to secrete.
- ACTH, which acts on their adrenal glands causing them to release the estrogen precursor **dehydroepiandrosterone sulfate** (**DHEAS**).
- This is converted into **estrogen** by the placenta.
- The rising level of estrogen causes the smooth muscle cells of the uterus to
  - synthesize **connexins** and form gap junctions. Gap junctions connect the cells electrically so that they contract together as labor begins.
  - express receptors for **oxytocin**.
- **Oxytocin** is secreted by the posterior lobe of the pituitary as well as by the uterus.
- **Prostaglandins** are synthesized in the placenta and uterus.
- The normal inhibition of uterine contraction by progesterone is turned off by several mechanisms while
- both oxytocin and prostaglandins cause the uterus to contract and labor begins.

Three or four days after the baby is born, the breasts begin to secrete milk. Milk synthesis is stimulated by the pituitary hormone **prolactin (PRL)**, and its release from the breasts is stimulated by **oxytocin**. Milk contains an **inhibitory peptide**. If the breasts are not fully emptied, the peptide accumulates and inhibits milk production. This autocrine action thus matches supply with demand.

Other Hormones

- **Relaxin**
  As the time of birth approaches in some animals (e.g., pigs, rats), this polypeptide has been found to:
  - relax the pubic ligaments
  - soften and enlarge the opening to the cervix

  Relaxin is found in pregnant humans but at higher levels early in pregnancy than close to the time of birth. Relaxin promotes angiogenesis, and in humans it probably plays a more important role in the development of the interface between the uterus and the placenta that it does in the birth process.

- **Activins**, **Inhibins**, **Follistatin**.

  These proteins are synthesized within the follicle. Activins and inhibins bind to follistatin. Activins increase the action of FSH; inhibins, as their name suggests, inhibit it. How important they are in humans remains to be seen. However the important role that activin and follistatin play in the embryonic development of vertebrates justifies mentioning them here.

Oral contraceptives - the "pill"

The feedback inhibition of GnRH secretion by estrogens and progesterone provides the basis for the most widely-used form of contraception. Dozens of different formulations of **synthetic estrogens** or **progestins** (progesterone relatives or both are available. Their **inhibition of GnRH** prevents the mid-cycle surge of LH and ovulation. Hence there is no egg to be fertilized. Usually the preparation is taken for about three weeks and then stopped long enough for normal menstruation to occur. The main side-effects of the pill stem from an increased tendency for blood clots to form (estrogen enhances clotting of the blood).

RU-486

RU-486 (also known as mifepristone) is a synthetic steroid related to *progesterone*. Unlike the synthetic progestins used in oral contraceptives that mimic the actions of progesterone, RU-486 is a progesterone *antagonist*; that is, it blocks the action of progesterone. It does this by binding more tightly to the *progesterone receptor* than progesterone itself but without the normal biological effects:

- The RU-486/receptor complex is not active as a transcription factor.
- Thus genes that are turned on by progesterone are turned off by RU-486.
- The proteins needed to establish and maintain pregnancy are no longer synthesized.
- The endometrium breaks down.
- The embryo detaches from it and can no longer make chorionic gonadotropin (**HCG**).
- Consequently the corpus luteum ceases its production of progesterone.
- The inhibition on uterine contraction is lifted.

- Soon the embryo and the breakdown products of the endometrium are expelled.

These properties of RU-486 have caused it to be used to **induce abortion** of an unwanted fetus. In practice, the physician assists the process by giving a synthetic prostaglandin (e.g., **misoprostol** [Cytotec®]) 36–48 hours after giving the dose of RU-486. Use of RU-486 is generally limited to the first seven weeks of pregnancy. RU-486 has been used for many years in some countries. However, the controversies surrounding abortion in the United States kept it from being authorized for use here until September 2000.

Menopause

The menstrual cycle continues for many years. But eventually, usually between 42 and 52 years of age, the follicles become less responsive to FSH and LH. They begin to secrete less estrogen. Ovulation and menstruation become irregular and finally cease. This cessation is called *menopause*.

With levels of estrogen now running one-tenth or less of what they had been, the hypothalamus is released from their inhibitory influence (bar). As a result it now stimulates the pituitary to increased activity. The concentrations of FSH and LH in the blood rise to ten or more times their former values. These elevated levels may cause a variety of unpleasant physical and emotional symptoms.

Hormone Replacement Therapy (HRT)

Many menopausal women elect to take a combination of estrogen and progesterone after they cease to make their own. The benefits are (1) reduction in the unpleasant symptoms of the menopause and (2) a reduction in the loss of calcium from bones and thus a reduction in osteoporosis and the fractures that accompany it. It was also believed that HRT reduced the risk of cardiovascular disease. However, a recent study of 16,000 menopausal women was stopped 3 years early when it was found that, in fact, HRT increased (albeit only slightly) not decreased the incidence of cardiovascular disease. Perhaps synthetic **selective estrogen response modulators** or SERMs (raloxifene is an example) will provide the protective effects without the harmful ones.

Environmental estrogens

Some substances that find their way into the environment, such as

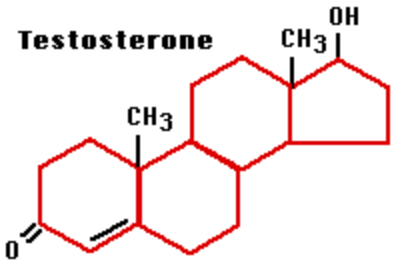
- **DDE**, a breakdown product of the once widely-used insecticide DDT
- **DDT** itself — still used in some countries (e.g., Mexico)
- **PCBs**, chemicals once used in a wide variety of industrial applications

can bind to the estrogen (and androgen) receptors and mimic (weakly) the effects of the hormone. This has created anxiety that they may be responsible for harmful effects such as cancer and low sperm counts.

However, there is as yet little evidence to support these worries. No epidemiological relationship has been found between the incidence of breast cancer and the levels of these compounds in the body. As for laboratory studies that found a synergistic effect of two of these substances on receptor binding (findings that created the great alarm), these have not been replicated in other laboratories, and the authors of the original report have since withdrawn it as invalid.

Males

The principal **androgen** (male sex hormone) is **testosterone**. This steroid is manufactured by the interstitial (Leydig) cells of the **testes**. Secretion of testosterone increases sharply at puberty and is responsible for the development of the so-called secondary sexual characteristics (e.g., beard) of men.



Testosterone is also essential for the production of sperm. Production of testosterone is controlled by the release of **luteinizing hormone** (**LH**) from the anterior lobe of the pituitary gland, which is in turn controlled by the release of GnRH from the hypothalamus. LH is also called interstitial cell stimulating hormone (ICSH).

Hypothalamus	→	GnRH	→	Pituitary	→	LH	→	Testes	→	Testosterone

The level of testosterone is under negative-feedback control: a rising level of testosterone suppresses the release of GnRH from the hypothalamus. This is exactly parallel to the control of estrogen secretion in females.

In mice, osteocalcin, a hormone secreted by osteoblasts of the bone, stimulates the synthesis of testosterone by Leydig cells even more powerfully than LH. Whether this effect occurs in humans remains to be seen.

Males need estrogen, too!

In 1994, a man was described who was homozygous for a mutation in the gene encoding the **estrogen receptor**. A single nonsense mutation had converted a codon (CGA) for arginine early in the protein into a STOP codon (TGA). Thus no complete estrogen receptor could be synthesized.

This man was extra tall, had *osteoporosis* and "knock-knees", but was otherwise well. His genetic defect confirms the important role that estrogen has in both sexes for normal bone development. It is not known whether this man was fertile or not. However, mutations in their estrogen receptor gene have been found in other men who are sterile, and male mice whose estrogen receptor gene has been "knocked out" are sterile.

Another function of estrogen in males. The accumulation of fat in the abdomen, so characteristic of aging males (including yours truly), is caused by declining levels of estrogen.

Anabolic steroids

A number of **synthetic androgens** are used for therapeutic purposes. These drugs promote an increase in muscle size with resulting increases in strength and speed. This has made them popular with some athletes, e.g., weight lifters, cyclists, runners, swimmers, professional football players. Usually these athletes (females as well as males) take doses far greater than those used in standard therapy. Such illicit use carries dangers (besides being banned from an event because of a positive drug test): acne, a decrease in libido, and — in males — testicle size and sperm counts to name a few.

Genetic abnormalities of gonadal function

Many things can go wrong with sexual development in both males and females, fortunately rarely. Let's look at a few that clearly result from the inheritance of single-gene mutations.

- Inherited mutations in both copies of the gene encoding the **GnRH receptor** result in failure to develop at puberty.
- Mutations in the gene encoding the **LH receptor** prevent normal sexual development in both sexes.
- Mutations in the gene encoding the **FSH receptor** block development of the gonads in both males and females.
- Mutations in any of the genes encoding the enzymes for synthesis and metabolism of **testosterone** interfere with normal sexual function in males.
- A similar spectrum of disorders in males can be caused by mutations in the genes encoding the **androgen receptor**.

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15.6.1.8: Progesterone

Progesterone is one of the steroid hormones. It is secreted by the corpus luteum and by the placenta and is responsible for preparing the body for pregnancy and, if pregnancy occurs, maintaining it until birth.

- **Corpus luteum**: Progesterone secretion by the corpus luteum occurs after ovulation and continues the preparation of the endometrium for a possible pregnancy. It also inhibits contraction of the uterus and inhibits development of a new follicle. If pregnancy does not occur, secretion wanes toward the end of the menstrual cycle, and menstruation begins.
- **Placenta**: If pregnancy does occur, the placenta begins to secrete progesterone which supplements that of the corpus luteum. In fact, by the fifth month of pregnancy, the placenta secretes sufficient progesterone by itself that the corpus luteum is no longer essential to maintain pregnancy.

Progesterone, like all steroids, is a small hydrophobic molecule. Thus it diffuses freely through the plasma membrane of all cells. However, in target cells, like those of the endometrium it becomes tightly bound to a cytoplasmic protein the progesterone receptor. The complex of receptor and its hormone moves into the nucleus. There it binds to a *progesterone response element*, which is a specific sequence of DNA in the promoters of certain genes that is needed to turn those genes on (or off). Thus the complex of progesterone with its receptor forms a transcription factor.

Progestins

Progestins are synthetic modifications of the progesterone molecule. Several different ones are prescribed

- for birth control pills
- for hormone replacement therapy (HRT) to reduce the unpleasant symptoms of the menopause
- to treat young women who cease to menstruate normally
- to prevent premature birth

Some examples:

- norgestrel (Trade name = Orvette®) - used as an oral contraceptive
- levonorgestrel
  - the ingredient in "Plan B", an oral contraceptive taken after unprotected intercourse.
  - released from Mirena®, an intrauterine device (IUD).
- norethindrone (Trade name = Aygestin®; used in HRT (hormone replacement therapy)

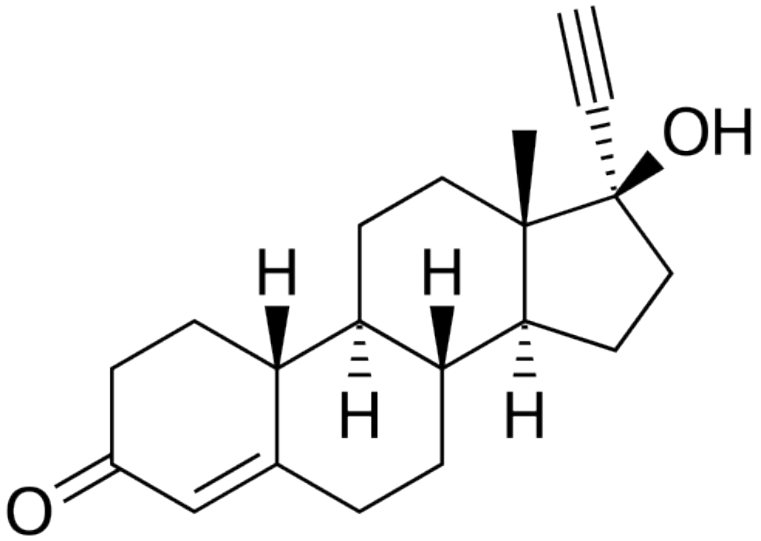


Figure 1: Norethisterone is one of the most widely used progestins. (Public Domain; Fvasconcellos).

RU-486

RU-486 (also known as mifepristone) is a synthetic steroid related to progesterone. Unlike the progestins discussed above, that mimic the action of progesterone, RU-486 blocks the action of progesterone. Synthetic molecules that mimic the action of a natural molecule are called agonists; those that oppose it are antagonists. RU-486 is a progesterone antagonist. It binds to the progesterone receptor, and in so doing prevents progesterone itself from occupying its receptor. Thus the gene transcription normally turned on by progesterone is blocked, and the proteins necessary to begin and maintain pregnancy are not synthesized. If RU-486 is taken shortly after intercourse, it prevents pregnancy. If taken early in pregnancy, it causes the embryo to be aborted. This result has caused RU-486 to be widely used in Europe to terminate early pregnancy. It has not found widespread acceptance in the U.S.

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15.6.1.9: Melatonin and the Pineal Gland

This page discusses the pineal gland's role in producing melatonin, a hormone essential for regulating sleep-wake cycles influenced by light and darkness. It notes that blind individuals may face disruptions in these cycles. Melatonin supplements can enhance sleep quality and help with jet lag, generally without severe side effects.

The pineal gland is a tiny structure located at the base of the brain. Its principal hormone is **melatonin**, a derivative of the amino acid tryptophan.

Synthesis and release of melatonin is

- **stimulated by darkness**
- **inhibited by light**.

But even without visual cues, the level of melatonin in the blood rises and falls on a daily (**circadian**) cycle with peak levels occurring in the wee hours of the morning. However, this cycle tends to drift in people who are totally blind — often making them sleepy during the day and wide awake at night. Giving melatonin at bedtime has proved helpful in a number of cases

Melatonin is readily available in drug stores and health food stores, and it has become quite popular. Ingesting even modest doses of melatonin raises the melatonin level in the blood to as much as 100 times greater than normal. These levels appear:

- to promote going to sleep and thus help insomnia
- to hasten recovery from jet lag
- not to have dangerous side effects

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15.6.1.10: Hormones of Kidney, Skin and Heart

Kidney

The human kidney secretes two hormones:

- Erythropoietin (**EPO**)
- **Calcitriol** (1,25[OH]2 Vitamin D3)

as well as the enzyme **renin**.

Erythropoietin (EPO)

Erythropoietin is a glycoprotein that acts on the bone marrow to increase the production of red blood cells. Stimuli such as bleeding or moving to high altitudes (where oxygen is scarcer) trigger the release of EPO. People with failing kidneys can be kept alive by dialysis, which only cleanses the blood of wastes. Without a source of EPO, these patients suffer from anemia. Now, thanks to recombinant DNA technology, **recombinant human EPO** is available to treat these patients.

Some other uses for recombinant EPO:

- Some of the drugs used to treat AIDS, zidovudine (AZT) for example, cause anemia as a side effect. Recombinant EPO helps AIDS patients cope with this one of the many problems that the disease creates.
- Recombinant EPO improves the anemia that is such a frequent side effect of cancer chemotherapy.
- Severe blood loss in Jehovah's Witnesses, whose religion forbids them to receive blood transfusions, can also be helped with recombinant EPO.

Because EPO increases the hematocrit, it enables more oxygen to flow to the skeletal muscles. Some cyclists (and distance runners) have used recombinant EPO to enhance their performance. Although recombinant EPO has exactly the same sequence of amino acids as the natural hormone, the sugars attached by the cells used in the pharmaceutical industry differ from those attached by the cells of the human kidney. This difference can be detected by a test of the athlete's urine.

Another problem: since recombinant EPO became available, over two dozen young competitive cyclists have died unexpectedly (usually during the night). Perhaps an EPO-induced increase in their hematocrit — leading to a reduction in heart rate — is responsible. Prolonged exposure to reduced oxygen levels (e.g., living at high altitude) leads to increased synthesis of EPO. In mice, and perhaps in humans, this effect is mediated by the skin. Mouse skin cells can detect low levels of oxygen ("hypoxia") and if this persists, blood flow to the kidneys diminishes leading to increased synthesis of EPO by them.

EPO is also synthesized by osteoblasts in mice that have been made anemic and in the brain when oxygen becomes scarce there (e.g., following a stroke), and helps protect neurons from damage. Perhaps recombinant human EPO will turn out to be useful for stroke victims as well.

Calcitriol

Calcitriol is 1,25[OH]2 Vitamin D3, the active form of vitamin D. It is derived from

- calciferol (vitamin D3) which is synthesized in skin exposed to the ultraviolet rays of the sun
- precursors ("vitamin D") ingested in the diet.

Calciferol in the blood is converted into the active vitamin in two steps:

- calciferol is converted in the **liver** into 25[OH] vitamin D3
- this is carried to the **kidneys** (bound to a serum globulin) where it is converted into calcitriol. This final step is promoted by the **parathyroid hormone** (**PTH**).

Calcitriol Action

Calcitriol acts on

- the cells of the intestine to promote the absorption of calcium and phosphate from food
- bone to mobilize calcium from the bone to the blood

Calcitriol enters cells and, if they contain receptors for it (intestine cells do), it binds to them. The calcitriol receptors are zinc-finger transcription factors. The receptor-ligand complex bind to its **response element**, the DNA sequence:

5' AGGTCAnnnAGGTCA 3'

This sequence of nucleotides (**n** can be any nucleotide) is found in the promoters of genes that are turned on by calcitriol. Once the hormone-receptor complex is bound to its response element, other transcription factors are recruited to the promoter and transcription of the gene(s) begins.

Deficiency disorders

Insufficient calcitriol prevents normal deposition of calcium in bone.

- In childhood, this produces the deformed bones characteristic of **rickets**.
- In adults, it produces weakened bones causing **osteomalacia**.

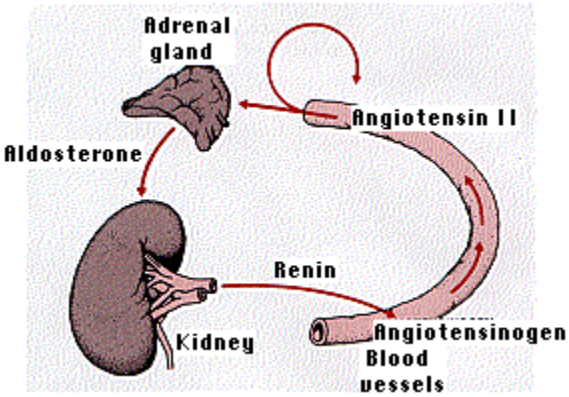


Figure 15.6.1.10.1 Angiotensin

Angiotensin II modulates all of the below actions to **increase in blood pressure:**

- constricts the walls of arterioles closing down capillary beds
- stimulates the proximal tubules in the kidney to reabsorb sodium ions
- stimulates the adrenal cortex to release **aldosterone**. Aldosterone causes the kidneys to reclaim still more sodium and thus water.
- increases the strength of the heartbeat
- stimulates the pituitary to release the **vasopressin**

Skin

When ultraviolet radiation strikes the skin, it triggers the conversion of dehydrocholesterol (a cholesterol derivative) into **calciferol** (vitamin D3). Calciferol travels in the blood to the liver where it is converted into 25[OH] vitamin D3. This compound travels to the kidneys where it is converted into **calcitriol** (1,25 $[OH]_2$ vitamin D3). This final step is promoted by the parathyroid hormone (PTH). Although called a vitamin, calciferol and its products fully qualify as hormones because they are

- made in certain cells
- carried in the blood
- affect gene transcription in target cells

Heart

Natriuretic Peptides

In response to a rise in blood pressure, the heart releases two peptides:

- A-type Natriuretic Peptide (ANP)

  This hormone of 28 amino acids is released from stretched **atria** (hence the "A").

- **B-type Natriuretic Peptide** (BNP)

  This hormone (29 amino acids) is released from the **ventricles**. (It was first discovered in brain tissue; hence the "B".)

Both hormones lower blood pressure by

- relaxing arterioles
- inhibiting the secretion of **renin** and **aldosterone**
- inhibiting the reabsorption of sodium ions by the kidneys.

The latter two effects reduce the reabsorption of water by the kidneys. So the volume of urine increases as does the amount of sodium excreted in it. The net effect of these actions is to reduce blood pressure by reducing the volume of blood in the circulatory system. These effects give ANP and BNP their name (natrium = sodium; uresis = urinate).

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15.6.1.11: Leptin - the Fat Hormone

This page discusses laboratory mice with genetic mutations ob/ob and db/db, which have enhanced understanding of leptin's role in appetite regulation and metabolism. Leptin, produced by fat cells, influences the hypothalamus, inhibits food intake, and affects liver and muscle cells. Although some human trials show potential benefits of recombinant leptin for certain genetic mutations, outcomes for the general obesity population have not been promising.

Leptin

Several strains of laboratory mice are homozygous for single-gene mutations that causes them to become grossly obese.

These fall into two classes:

- When *ob/ob* mice are treated with injections of leptin they lose their excess fat and return to normal body weight.
- db/db = mutations in the gene that encodes the **receptor** for leptin

Study of these animals has led to an understanding of the action of leptin in humans.

Human leptin is a protein of 167 amino acids. It is manufactured in the **adipocytes** (fat cells) of white adipose tissue, and the level of circulating leptin is directly proportional to the total amount of fat in the body.

Leptin acts on receptors in the hypothalamus of the brain where it:

- counteracts the effects of **neuropeptide Y** (a potent feeding stimulant secreted by cells in the gut and in the hypothalamus)
- counteracts the effects of **anandamide** (another potent feeding stimulant that binds to the same receptors as **THC**, the active ingredient of marijuana)
- promotes the synthesis of **α-MSH**, an appetite suppressant
- the result is the inhibition of food intake

This inhibition is long-term, in contrast to

- the rapid inhibition of eating by cholecystokinin (CCK)
- the slower suppression of hunger between meals mediated by PPY_{3-36}

The absence of a functional hormone (or its receptor) leads to uncontrolled food intake and resulting obesity.

Leptin also acts on hypothalamic neurons responsible for

- the conversion of white adipose tissue (WAT) into "beige" adipose tissue. "Beige" cells metabolize food into heat rather than storing it as fat. In mice, leptin promotes weight loss even with normal food intake.
- the secretion of gonadotropin-releasing hormone (**GnRH**). Women who are very thin from limited food intake or intense physical training may cease to menstruate because of their lack of leptin-secreting fat cells. Treating them with recombinant human leptin can sometimes restore normal menstruation.
- stimulating the sympathetic nervous system to trigger the breakdown of fat in adipose tissue.

In addition to its effect on the hypothalamus, leptin acts directly on the cells of the liver and skeletal muscle where it stimulates the oxidation of fatty acids in the mitochondria. This reduces the storage of fat in those tissues. T cells where it enhances the production of Th1 cells promoting inflammation. Mice without leptin are protected from autoimmune disease (which may account for the reports that restricting food intake helps humans with rheumatoid arthritis).

Mutations in the gene for leptin, or in its receptor, are rarely found in obese people.

The rare cases:

- Extreme obesity in five members of two families that are homozygous for mutations (frameshift in one family, missense in the other) in their **leptin** gene; i.e., they are like ***ob/ob*** mice.
- Extreme obesity among three members of a family that are homozygous for mutations in their **leptin receptor** gene; i.e., they

are like ***db/db*** mice.
- Only moderate obesity in people who are heterozygous (one mutant and one normal) for their leptin genes.

Recombinant human leptin is now available, but trials to see if it can reduce obesity in humans as it does in *ob/ob* mice have been disappointing.

However, the 16 September 1999 issue of **The New England Journal of Medicine** reported the results of a year-long trial of **recombinant human leptin** in a 9-year-old girl who is homozygous for a frameshift mutation in her leptin genes. The findings:

- She began the trial weighing 208 pounds (94.4 kg), of which 123 lbs (55.9 kg) was fat (adipose tissue).
- She was given daily injections of recombinant leptin for one year.
- At the end of that time,
- she had lost 36 lbs (16.4 kg), most of it fat.
- Her appetite and thus food intake had decreased.
- Her immune system made antileptin antibodies but these did not seem to interfere with the action of the hormone.

But trials of recombinant leptin in obese humans who do not have mutations in **both** their leptin genes have not shown any great benefit in weight reduction.

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15.6.1.12: Hormones of the Liver

This page details the liver's production of key hormones: Insulin-like Growth Factor-1 (IGF-1), angiotensinogen, thrombopoietin (TPO), hepcidin, and betatrophin. IGF-1, important for cell growth, peaks during puberty, while angiotensinogen is vital for blood pressure regulation. TPO regulates platelet production by stimulating bone marrow megakaryocytes. These hormones are essential for growth, maintaining blood pressure, and facilitating blood clotting.

The liver synthesizes and secretes at least four important hormones:

- **Insulin-like Growth Factor-1** (**IGF-1**)
- **Angiotensinogen**
- **Thrombopoietin**
- **Hepcidin**
- **Betatrophin**

Insulin-like Growth Factor-1

This protein of 70 amino acids was once called **somatomedin** because it is not growth hormone but is the immediate stimulus for growth of the body. Growth hormone released from the anterior lobe of the pituitary binds to receptors on the surface of liver cells which stimulates the synthesis and release of IGF-1 from them. Many cells have **receptors** for IGF-1, especially cells in the bone marrow and in the cartilaginous growing regions of the long bones.

Binding of IGF-1 to cells with receptors for it stimulates them to move from G1 of the cell cycle to S phase and on to mitosis.

Mice with one of their *Igf-1*receptor genes "knocked out" live 25% longer than normal mice. This may result from an increase in their resistance to the damaging effects of reactive oxygen species (ROS) or to an increased efficiency at clearing away clumped proteins in their cells (or both). These heterozygous mice appear to be normal in every other respect.

The levels of IGF-1 in the blood are highest during the years of puberty which is, of course, a time of rapid growth. Occasionally children are found that have stunted growth because they have inherited mutant genes for the **growth hormone (GH) receptor**. **Recombinant** human IGF-1 has been successfully used to treat them.

Angiotensinogen

This protein is released into the blood where it serves as the precursor for **angiotensin**. How angiotensin is manufactured, and the role it plays in maintaining blood pressure, is described in the discussion of renin.

Thrombopoietin (TPO)

Thrombopoietin is a protein of 332 amino acids. It stimulates precursor cells in the bone marrow to differentiate into **megakaryocytes**. Megakaryocytes generate **platelets**, essential to blood clotting.

The production of megakaryocytes — and thus platelets — is under homeostatic control. It works like this:

- Circulating platelets are covered with receptors for TPO.
- So are megakaryocytes and their precursors, but there are fewer of them.
- When platelet counts are high, most of the circulating TPO is bound to the platelets and less is left to stimulate megakaryocytes.
- When platelet counts drop, more TPO becomes available to stimulate megakaryocytes to replenish the platelet supply.
- Humans manufacture about 10^{11} platelets each day under normal conditions.

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15.6.1.13: Melanocyte Stimulating Hormone (MSH)

Melanocyte-stimulating hormone gets its name because of its effect on melanocytes: skin cells that contain the black pigment, **melanin**. In humans, melanocytes are responsible for moles, freckles, and suntan (and, if they turn cancerous, melanoma). In most vertebrates, MSH is produced by an intermediate lobe of the **pituitary gland**. Its secretion causes a dramatic darkening of the skin of fishes, amphibians, and reptiles. The darkening occurs as granules of melanin spread through the branches of specialized melanocytes called **melanophores**.

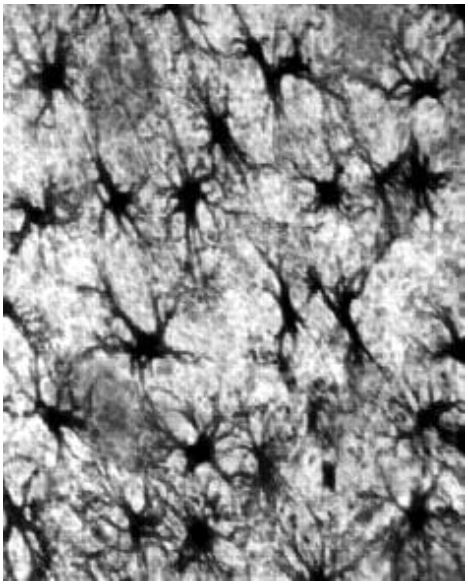


Figure 15.6.1.13.1 Melanocytes

The photomicrograph on the right shows melanophores in the skin of a frog with the melanin dispersed throughout the branches of the cells. This effect is produced by MSH. When the pigment retreats to the center of the cells, the skin lightens.

- The granules are carried outward along microtubules using kinesin as the motor.
- They assemble at the actin-rich periphery of the cell carried by a myosin.
- The granules are carried back to the center of the cell along microtubules using dynein as the motor.

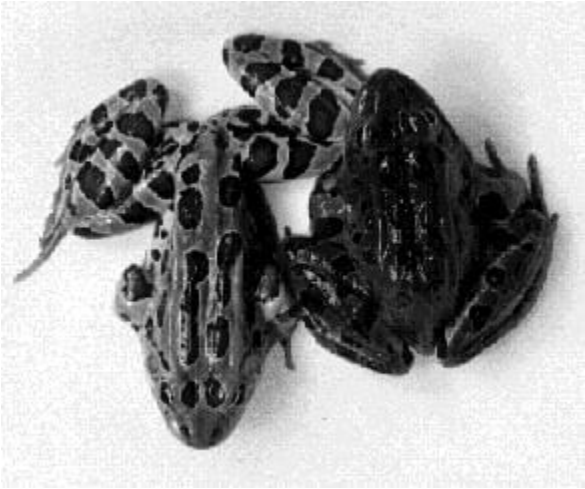


Figure 15.6.1.13.2 Frog with MSH

The above photo was taken a few moments after the frog on the right was injected with a small dose of MSH. The response to MSH does not occur during mitosis; presumably the microtubules with their dyneins and kinesins are needed for operation of the

mitotic spindle.

Tanning

Proopiomelanocortin (**POMC**), the same precursor molecule from which the adrenocorticotropic hormone (ACTH) is synthesized, also produces two forms of MSH. One of them, **α-MSH**, is identical to the first 13 amino acids at the amino terminal of ACTH. **α-MSH** is responsible for tanning in humans.

- When ultraviolet light strikes skin cells (keratinocytes), it activates the transcription factor p53.
- p53 turns on transcription of the gene encoding POMC.
- Cleavage of the POMC protein produces
  - **α-MSH**. This is secreted from the cells and stimulates nearby melanocytes (thus a paracrine effect) to synthesize **melanin** in packets called melanosomes. The melanosomes are transferred to the skin cells where they form a protective cap over the nucleus. This cap helps protect the DNA within the nucleus from the damaging effects of ultraviolet radiation.
  - ACTH. This is secreted into the blood and may help reduce skin inflammation by stimulating the release of glucocorticoids from the adrenal cortex.
  - **β-endorphin**. This **may** help suppress the pain of sunburn. In mice (and perhaps humans) the rise in the level of β-endorphin upon exposure to uv light activates mu (μ) receptors — the same ones that opiates bind to. This leads to similar addictive behavior - the mice seek uv exposure and show signs of withdrawal when β-endorphin binding is blocked (by naloxone).

Injections of a synthetic version of α-MSH called **Melanotan I** also darkens the skin. This raises the possibility of using melanotan to get a suntan without the risks of exposure to ultraviolet light. A second synthetic version of MSH dubbed Melanotan II also darkened the skin of male volunteers. Unexpectedly, it also caused many of them to develop penile erections. This has raised the possibility of using MSH to cure impotence.

MSH and appetite

Humans have no intermediate lobe in their pituitary gland, and MSH may not be a circulating hormone for us. However, **α-MSH** is produced by POMC neurons in the brain where it acts to **suppress appetite**. Some cases of extreme obesity have been traced to mutations in the brain **receptor** for α-MSH. Presumably these people are unable to respond to the appetite-suppressing effect of their α-MSH.

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15.6.2: Insect Hormones

Because of their rigid exoskeleton, insects can only grow by periodically shedding their exoskeleton - called **molting**. Molting occurs repeatedly during larval development. At the final molt, the adult emerges. In several insect orders, notably

- ants and bees (Hymenoptera) — example the honeybee
- flies (Diptera) — example *Drosophila melanogaster*
- butterflies and moths (Lepidoptera) such as the silkworm moth

the adult looks entirely different from the larva that preceded it. This marked transformation is called **complete metamorphosis**.

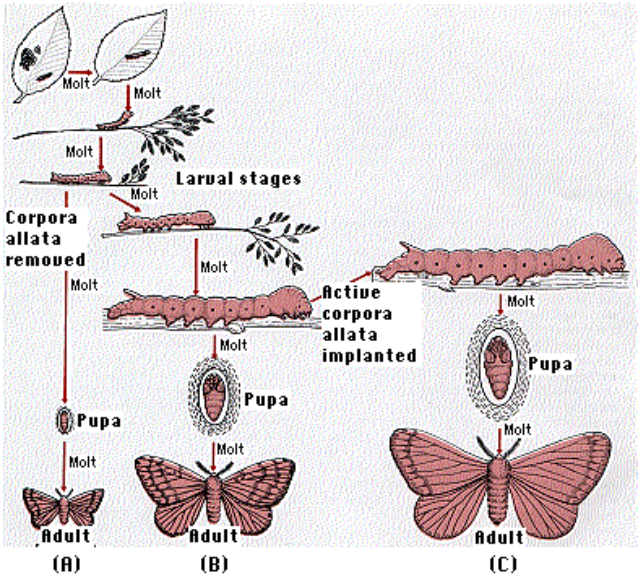


Figure 15.6.2.1 Development cycles of silkworm moth

Complete metamorphosis takes place during a seemingly-dormant stage called the **pupa**. In fact, intense biological activity is going on within the pupal case. The cells of virtually all the differentiated larval structures muscles, salivary glands, gut, etc. die by apoptosis. The nutrients they release are then available for the further development of nests of cells - the **imaginal discs** - that have been quietly developing within the larval body. Their differentiation produces the structures of the adult - legs, wings, compound eyes, etc.

The sequence ending in the center panel **(B)** shows the larval, pupal, and adult stages during normal development of the domestic silkworm moth, *Bombyx mori*.

Prothoracicotropic Hormone (PTTH)

Molting and pupation require the hormone, PTTH, secreted by a two pairs of cells in the brain of the larva. If these cells are cut out of the brain of a full-grown larva, pupation does not occur. This is not because of the trauma of surgery; if transplanted somewhere else in the caterpillar's body, pupation occurs normally. PTTH is a homodimer of two polypeptides of 109 amino acids. PTTH does not drive pupation directly but, as its name suggests, acts on the **prothoracic glands**.

Ecdysone

There are two prothoracic glands located in the thorax. Under the influence of PTTH, they secrete the steroid hormone **ecdysone**. Acting together, PTTH and ecdysone trigger every molt: larva-to-larva as well as pupa-to-adult. What, then, accounts for the dramatic changes of metamorphosis?

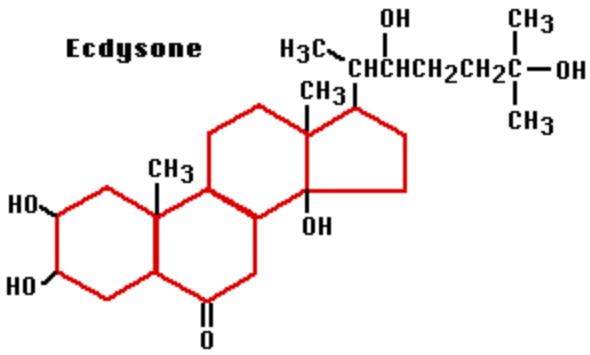


Figure 15.6.2.2 ECdysone

Juvenile Hormone (JH)

Juvenile hormone is secreted by two tiny glands behind the brain, the **corpora allata**. As long as there is enough JH, ecdysone promotes larva-to-larva molts. With lower amounts of JH, ecdysone promotes pupation. Complete absence of JH results in formation of the adult.

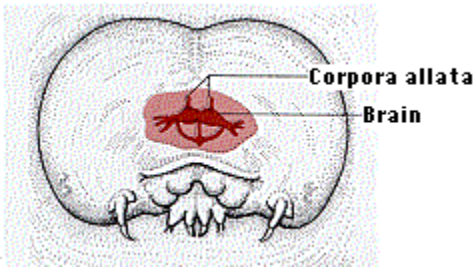


Figure 15.6.2.3 Corpora allata

So if the corpora allata are removed from an immature silkworm, it immediately spins a cocoon and becomes a small pupa. A miniature adult eventually emerges (shown in panel **(A)** above). Conversely, if the corpora allata of a young silkworm are place in the body of a fully-mature larva, metamorphosis does not occur. The next molt produces an extra-large caterpillar (panel **(C)** above).

JH affects gene expression

Adult insects do not normally molt, but if extra amounts of **PTTH** are given to an adult **Rhodnius** (the "kissing bug"), it is forced into an extra molt. The English insect physiologist V. B. Wigglesworth showed that if juvenile hormone is first applied to the insect's exoskeleton, the regions affected by it revert to larval type after this extra molt.

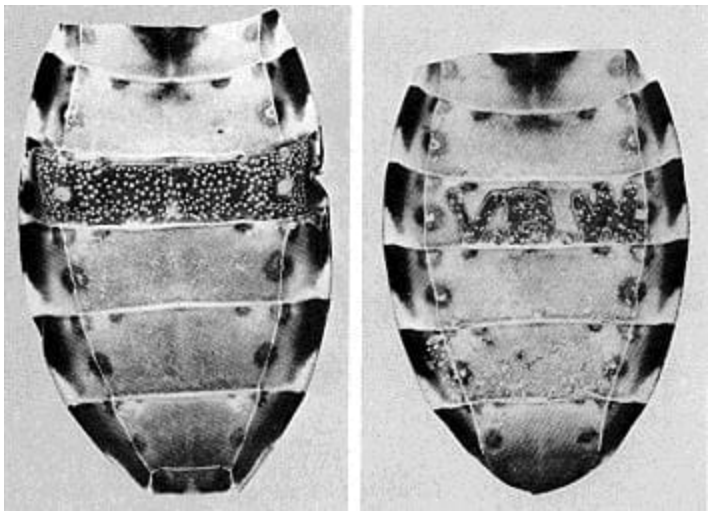


Figure 15.6.2.4 Juvenile hormone applied to insects. These images (courtesy of Dr. Wigglesworth) show his results. Left: application of a band of juvenile hormone to the cuticle of an adult Rhodnius results in the formation of larval cuticle (speckled band) when the insect is forced to undergo an extra molt. Right: Dr. Wigglesworth has printed his initials with juvenile hormone.

What a beautiful example of the power of a single molecule to unleash a different pattern of gene expression! Presumably, JH interacts with hormone receptors in the cells to produce a new set of transcription factors.

Insect hormones and pest control

Knowledge of insect hormones has provided a number of opportunities to enlist them or molecules related to them in the battle against insect pests.

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15.7: SEXUAL REPRODUCTION

Sexual reproduction is the formation of a new individual following the union of two **gametes**. In humans and the majority of other eukaryotes plants as well as animals the two gametes differ in structure ("anisogamy") and are contributed by different parents. Gametes need motility to be able to meet and unite and food to nourish the developing embryo. In animals (and some plants), these two rather contrasting needs are met by anisogametes: sperm that are motile (and small) and eggs that contain food.



-  Topic hierarchy 

15.7A: Sexual Reproduction

15.7B: Asexual Reproduction in Animals

15.7C: Birth Control

15.7D: Prenatal Screening

15.7E: Extraembryonic Membranes and the Physiology of the Placenta

15.7F: Genetic Mosaics

15.7G: Human Cloning

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15.7A: Sexual Reproduction

Sexual reproduction is the formation of a new individual following the union of two **gametes**. In humans and the majority of other eukaryotes plants as well as animals the two gametes differ in structure ("anisogamy") and are contributed by different parents. Gametes need motility to be able to meet and unite and food to nourish the developing embryo. In animals (and some plants), these two rather contrasting needs are met by anisogametes: sperm that are motile (and small) and eggs that contain food.

Sex Organs of the Human Male

The reproductive system of the male has two major functions: (1) production of sperm and (2) delivery of these to the reproductive tract of the female. Sperm production - spermatogenesis - takes place in the **testes**. Each testis is packed with **seminiferous tubules** (laid end to end, they would extend more than 20 meters) where spermatogenesis occurs.

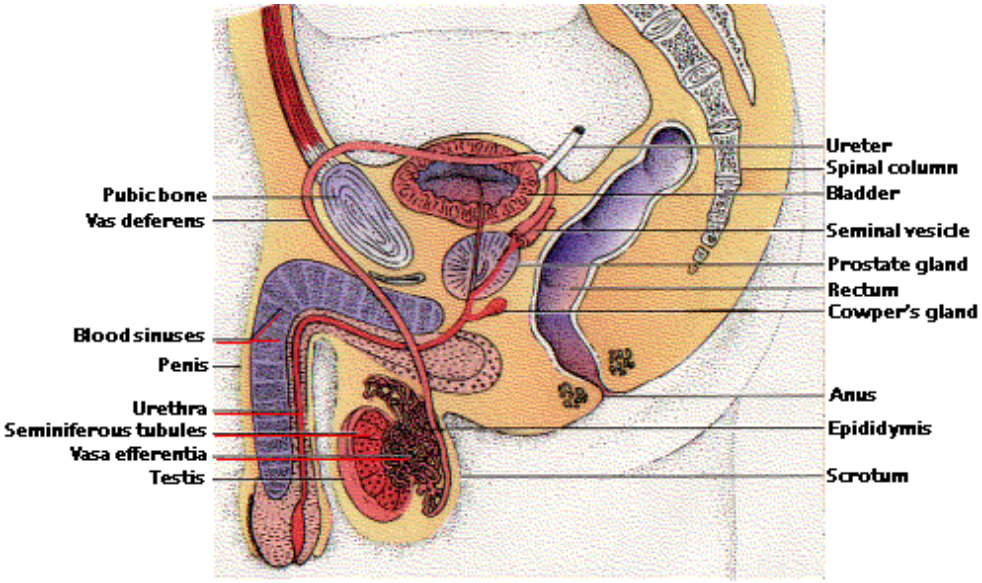


Figure 15.7.1.1 Male reproductive system

Spermatogenesis

The walls of the seminiferous tubules consist of diploid spermatogonia, stem cells that are the precursors of sperm. **Spermatogonia divide by mitosis to produce more spermatogonia or** differentiate into **spermatocytes**. Meiosis of each spermatocyte produces 4 haploid **spermatids**. This process takes over three weeks to complete. Then the spermatids differentiate into sperm, losing most of their cytoplasm in the process.

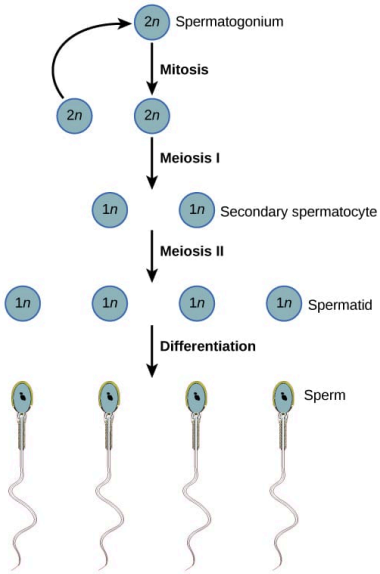


Figure 15.7.1.2 Spermatogenesis. During spermatogenesis, four sperm result from each primary spermatocyte, which divides into two haploid secondary spermatocytes; these cells will go through a second meiotic division to produce four spermatids. (CC BY; OpenStax).

For simplicity, the figure shows the behavior of just a single pair of homologous chromosomes with a single crossover. With 22 pairs of autosomes and an average of two crossovers between each pair, the variety of gene combinations in sperm is very great.

Sperm

Sperm cells are little more than flagellated nuclei. Each consists of a head, which has an **acrosome** at its tip and contains a haploid set of chromosomes in a compact, inactive, state, a **midpiece** containing mitochondria and a single centriole, and a **tail** which is a *flagellum*. This electron micrograph (courtesy of Dr. Don W. Fawcett and Susumu Ito) shows the sperm cell of a bat. Note the orderly arrangement of the mitochondria. They supply the ATP to power the whiplike motion of the tail.

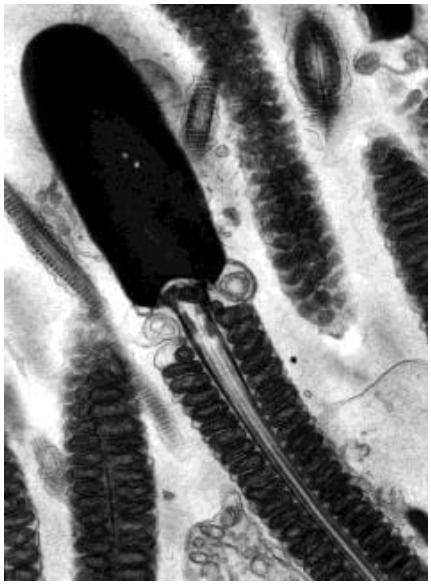


Figure 15.7.1.3 Bat sperm

An adult male manufactures over 100 million sperm cells each day. These gradually move into the **epididymis** where they undergo further maturation. The acidic environment in the epididymis keeps the mature sperm inactive. In addition to making sperm, the testis is an **endocrine gland**. Its principal hormone, **testosterone**, is responsible for the development of the secondary sex characteristics of men such as the beard, deep voice, and masculine body shape. **Testosterone is also essential for making sperm.** Testosterone is made in the **interstitial cells** (also called Leydig cells) that lie between the seminiferous tubules.

LH

Interstitial cells are, in turn, the targets for a hormone often called interstitial cell stimulating hormone (**ICSH**). It is a product of the anterior lobe of the pituitary gland. However, ICSH is identical to the luteinizing hormone (**LH**) found in females, and I prefer to call it LH.

FSH

Follicle-stimulating hormone (also named for its role in females) acts directly on spermatogonia to stimulate sperm production (aided by the LH needed for testosterone synthesis).

Sex Organs of the Human Female

The responsibility of the female mammal for successful reproduction is considerably greater than that of the male.

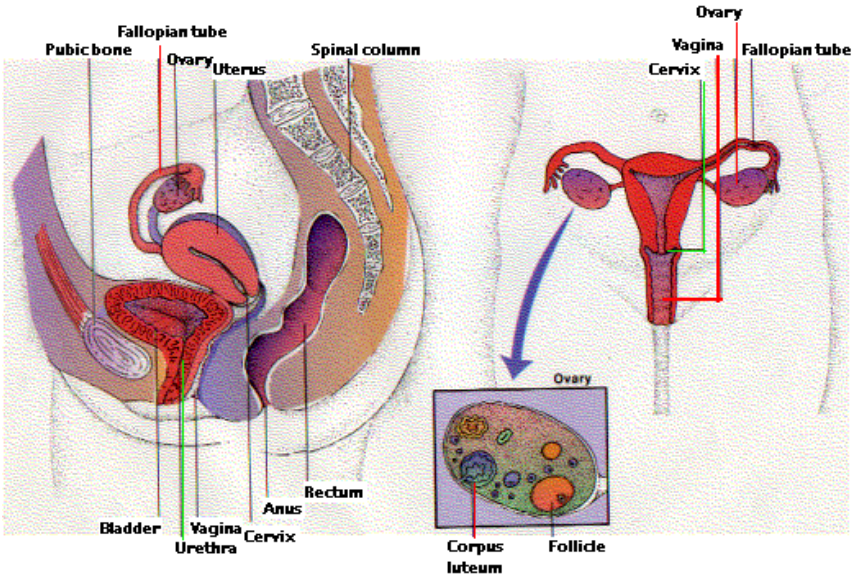


Figure 15.7.1.4 Female reproductive system

She must

- manufacture eggs
- be equipped to receive sperm from the male
- provide an environment conducive to fertilization and implantation
- nourish the developing baby not only before birth but after too

Oogenesis

Egg formation takes place in the **ovaries**. In contrast to males, the initial steps in egg production occur prior to birth. Diploid stem cells called **oogonia** divide by mitosis to produce more oogonia and **primary oocytes**. By the time the fetus is 20 weeks old, the process reaches its peak and all the oocytes that she will ever possess (~4 million of them) have been formed (*). By the time she is born, only about 1 million of these remain (the others eliminated by apoptosis). Each has begun the first steps of the first meiotic division stopping at the diplotene stage of meiosis I.

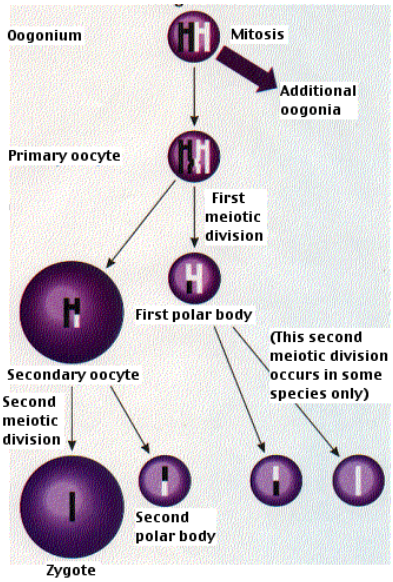


Figure 15.7.1.5 Oogenesis

No further development occurs until years later when the girl becomes sexually mature. Then the primary oocytes recommence their development, usually one at a time and once a month. The primary oocyte grows much larger and completes meiosis I, forming a large **secondary oocyte** and a small **polar body** that receives little more than one set of chromosomes. Which chromosomes end up in the egg and which in the polar body is entirely a matter of chance.

In humans (and most vertebrates), the first polar body does not go on to meiosis II, but the secondary oocyte does proceed as far as **metaphase** of meiosis II and then stops.

Only if **fertilization** occurs will meiosis II ever be completed. Entry of the sperm restarts the cell cycle breaking down MPF (M-phase promoting factor) and turning on the anaphase-promoting complex (APC). Completion of meiosis II converts the secondary oocyte into a fertilized egg or zygote (and also a second polar body). As in the diagram for spermatogenesis, the behavior of the chromosomes is greatly simplified.

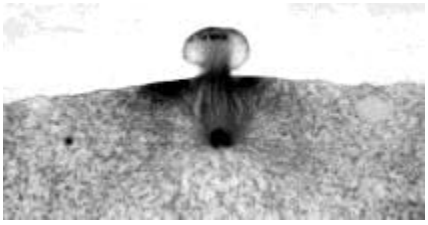


Figure 15.7.1.6 Polar body formation during oogenesis

The photomicrograph (courtesy of Turtox) shows polar body formation during oogenesis in the whitefish. Even allowing for the fact that fish eggs are larger than mammalian eggs, you can readily see how the polar body gets little more than one set of chromosomes.

These events take place within a **follicle**, a fluid-filled envelope of cells surrounding the developing egg. The ripening follicle also serves as an **endocrine gland**. Its cells make a mixture of steroid hormones collectively known as **estrogen**. Estrogen is responsible for the development of the secondary sexual characteristics of a mature woman, e.g.,

- a broadening of the pelvis
- development of the breasts
- growth of hair around the genitals and in the armpits
- development of adipose tissue leading to the more rounded body contours of adult women.

Estrogen continues to be secreted throughout the reproductive years of women During this period, it plays an essential role in the monthly **menstrual cycle**.

Ovulation

Ovulation occurs about two weeks after the onset of menstruation. In response to a sudden surge of LH, the follicle ruptures and discharges a secondary oocyte. This is swept into the open end of the **fallopian tube** and begins to move slowly down it.

Copulation and Fertilization

For fertilization to occur, sperm must be deposited in the vagina within a few (5) days before or on the day of ovulation. Sperm transfer is accomplished by copulation. Sexual excitation dilates the arterioles supplying blood to the penis. Blood accumulates in three cylindrical spongy sinuses that run lengthwise through the penis. The resulting pressure causes the penis to enlarge and erect and thus able to penetrate the vagina. Movement of the penis back and forth within the vagina causes sexual tension to increase to the point of **ejaculation**. Contraction of the walls of each **vas deferens** propels the sperm along. Fluid is added to the sperm by the **seminal vesicles**, Cowper's glands, and the **prostate gland**. These fluids provide

- a source of energy (fructose)
- an alkaline environment to activate the sperm
- perhaps in other ways provide an optimum chemical environment for them

The mixture of sperm and accessory fluids is called **semen**. It passes through the **urethra** and is expelled into the vagina. Physiological changes occur in the female as well as the male in response to sexual excitement, although these are not as readily apparent. In contrast to the male, however, such responses are not a prerequisite for copulation and fertilization to occur. Once deposited within the vagina, the sperm proceed on their journey into and through the uterus and on up into the fallopian tubes. It is here that fertilization may occur if an "egg" is present (strictly speaking, it is still a secondary oocyte until after completion of meiosis II).

Although sperm can swim several millimeters each second, their trip to and through the fallopian tubes may be assisted by muscular contraction of the walls of the uterus and the tubes. The trip is fraught with heavy mortality. An average human ejaculate contains over one hundred million sperm, but only a few dozen complete the journey, arriving within 15 minutes of ejaculation. And of these, only one will succeed in fertilizing the egg.

Sperm swim towards the egg by chemotaxis following a gradient of progesterone secreted by cells surrounding the egg. Progesterone opens CatSper ("cation sperm") channels in the plasma membrane surrounding the anterior portion of the sperm tail. This allows an influx of Ca^{2+} ions which causes the flagellum to beat more rapidly and vigorously. Fertilization begins with the binding of a sperm head to the glycoprotein coating of the egg (called the **zona pellucida**). Exocytosis of the **acrosome** at the tip of the sperm head releases enzymes that digest a path through the zona and enable the sperm head to bind to the plasma membrane of the egg. The binding is mediated by the binding of two membrane proteins:

- Izumo1 on the surface of the sperm
- Juno, its receptor on the egg surface

Fusion of their respective membranes allows the entire contents of the sperm to be drawn into the cytosol of the egg. Even though the sperm's mitochondria enter the egg, they are almost always destroyed by autophagy and do not contribute their genes to the embryo. So human mitochondrial DNA is almost always inherited from mothers only. Within moments, enzymes released from the egg cytosol act on the zona making it impermeable to other sperm that arrive. Soon the nucleus of the successful sperm enlarges into the **male pronucleus**. At the same time, the egg (secondary oocyte) completes meiosis II forming a **second polar body** and the **female pronucleus**.

The male and female pronuclei move toward each other while duplicating their DNA in S phase. Their nuclear envelopes disintegrate. A spindle is formed (following replication of the sperm's centriole), and a full set of dyads assembles on it. The fertilized egg or **zygote** is now ready for its first mitosis. When this is done, 2 cells each with a diploid set of chromosomes are formed.

In sea urchins, the block to additional sperm entry and the fusion of the pronuclei are triggered by nitric oxide generated in the egg by the sperm acrosome.

Pregnancy

Development begins while the fertilized egg is still within the **fallopian tube**. Repeated mitotic divisions produces a solid ball of cells called a **morula**. Further mitosis and some migration of cells converts this into a hollow ball of cells called the **blastocyst**. Approximately one week after fertilization, the blastocyst embeds itself in the thickened wall of the uterus, a process called **implantation**, and pregnancy is established.

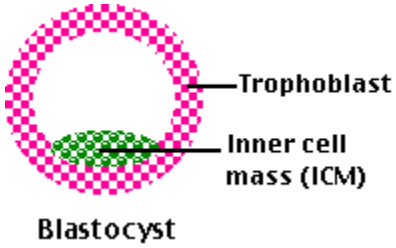


Fig.15.7.1.7 Blastocyst

The blastocyst produces two major collections of cells:

- Three or four blastocyst cells develop into the **inner cell mass**, which will form 3 extraembryonic membranes: **amnion**, **yolk sac**, and (a vestigial) **allantois** and in about 2 months, become the fetus and, ultimately, the baby.
- The remaining 100 or so cells form the **trophoblast**, which will develop into the **chorion** that will go on to make up most of the **placenta**. All the extraembryonic membranes play vital roles during development but will be discarded at the time of birth.

The placenta grows tightly fused to the wall of the uterus. Its blood vessels, supplied by the fetal heart, are literally bathed in the mother's blood. Although there is normally no mixing of the two blood supplies, the placenta does facilitate the transfer of a variety of materials between the fetus and the mother such as

- receiving food
- receiving oxygen and discharging carbon dioxide
- discharging urea and other wastes
- receiving **antibodies** (chiefly of the IgG class). These remain for weeks after birth, protecting the baby from the diseases to which the mother is immune.

But the placenta is not simply a transfer device. Using raw materials from the mother's blood, it synthesizes large quantities of proteins and also some hormones. The metabolic activity of the placenta is almost as great as that of the fetus itself. The **umbilical cord** connects the fetus to the placenta. It receives deoxygenated blood from the iliac arteries of the fetus and returns oxygenated blood to the liver and on to the inferior vena cava.

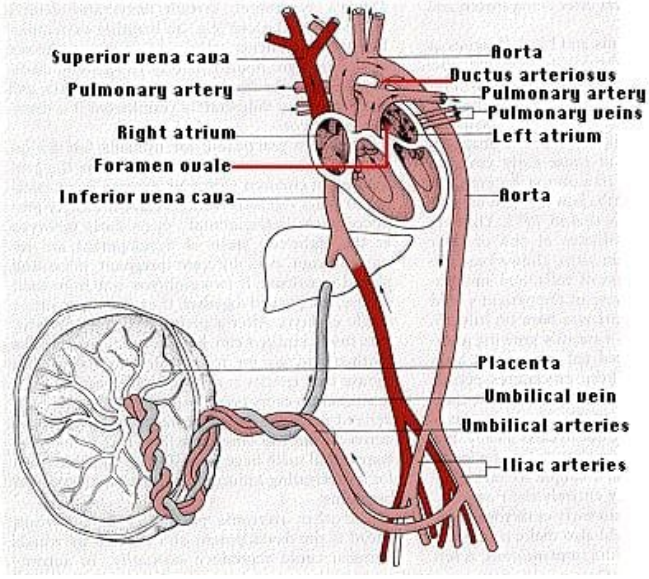


Figure 15.7.1.8 Foramen ovale

Because its lungs are not functioning, circulation in the fetus differs dramatically from that of the baby after birth. While within the uterus, blood pumped by the right ventricle bypasses the lungs by flowing through the **foramen ovale** and the **ductus arteriosus**.

Although the blood in the placenta is in close contact with the mother's blood in the uterus, intermingling of their blood does not normally occur. However, some of the blood cells of the fetus usually do escape into the mother's circulation — where they have been known to survive for decades. This raises the possibility of doing **prenatal diagnosis** of genetic disorders by sampling the mother's blood rather than having to rely on the more invasive procedures of amniocentesis and chorionic villus sampling (CVS).

Fragments of fetal DNA (~ 300 bp long) from apoptotic cells of the placenta are also found in the mother's plasma as early as 5 weeks after implantation. These can be tested for various forms of aneuploidy, e.g. the trisomy 21 of Down syndrome. Far rarer is the leakage of mother's blood cells into the fetus. However, it does occur. A few pregnant women with leukemia or lymphoma have transferred the malignancy to their fetus. Some babies have also acquired melanoma from the transplacental passage of these highly-malignant cells from their mother.

During the first 2 months of pregnancy, the basic structure of the baby is being formed. This involves cell division, cell migration, and the differentiation of cells into the many types found in the baby. During this period, the developing baby - called an **embryo** - is very sensitive to anything that interferes with the steps involved. Virus infection of the mother, e.g., by rubella ("German measles") virus or exposure to certain chemicals may cause malformations in the developing embryo. Such agents are called **teratogens** ("monster-forming"). The tranquilizer, thalidomide, taken by many pregnant European women between 1954 and 1962, turned out to be a potent teratogen and was responsible for the birth of several thousand deformed babies.

After about two months, all the systems of the baby have been formed, at least in a rudimentary way. From then on, development of the **fetus**, as it is now called, is primarily a matter of growth and minor structural modifications. The fetus is less susceptible to teratogens than is the embryo. Pregnancy involves a complex interplay of **hormones**.

The placenta is an allograft

One of the greatest unsolved mysteries in immunology is how the placenta survives for 9 months without being rejected by the mother's immune system. Every cell of the placenta carries the father's genome (a haploid set of his chromosomes); including one of his #6 chromosomes where the genes for the **major histocompatibility antigens** (HLA) are located.

One partial exception: none of the genes on the father's X chromosome are expressed. While X-chromosome inactivation is random in the cells of the fetus, it is NOT random in the cells of the trophoblast. In every cell of the trophoblast — and its descendants — it is the paternal X chromosome that is inactivated. But this does not solve our problem because the genes for all the major histocompatibility antigens are located on chromosome 6, which is not inactivated.

Thus the placenta is immunologically as foreign to the mother as a kidney transplant would be. Yet it thrives. Despite a half-

century of research, the mechanism for this immunologically privileged status remains uncertain. But one thing is clear - The mother is **not** intrinsically tolerant of the father's antigens. She will promptly reject a skin transplant from the father. She develops antibodies against his histocompatibility antigens expressed by the fetus. In fact, women who have borne several children by the same father are often excellent sources of anti-HLA serum for use in tissue typing. So what accounts for the phenomenon? Some possibilities:

- The placenta does not express class II histocompatibility antigens.
- Nor does it express the strongly-immunogenic class I histocompatibility antigens (HLA-A, HLA-B). It does express HLA-C, but this is only weakly immunogenic.
- The cells of the placenta secrete progesterone, which is immunosuppressive.
- In lab rats the embryos (and the mother's endometrium) secrete corticotropin-releasing hormone (CRH). This hormone induces the expression of Fas ligand (**FasL**) on the cells of the placenta. Activated T cells express **Fas** so any threatening T cells would commit suicide by apoptosis when they encounter FasL on their target.
- In laboratory mice the cells of the placenta degrade the amino acid tryptophan. Tryptophan is essential for T-cell function. When mice are treated with an inhibitor of the Trp-degrading enzyme, their fetuses are promptly aborted by the action of the mother's lymphocytes. (D. H. Munn, et. al., **Science**, **281:** 1191, 21 Aug 1998.)
- In mice, expression of genes encoding cytokines needed to attract effector T cells (e.g., CTLs) into a tissue is suppressed in the cells of the placenta.
- Perhaps most important of all is the increased production in the mother of immunosuppressive **regulatory T cells** (Treg).
    - Depletion of Treg cells in pregnant mice leads to spontaneous abortion while
    - injection of Treg cells into mice that are otherwise prone to abortion enables them to carry their fetuses to term.
    - In humans, the number of Treg cells rises during pregnancy (in the fetus as well as the mother).

In vitro fertilization

On July 25, 2013 Louise Brown celebrated her 35th birthday. She was the first of what today number some four million (worldwide) "test tube babies"; that is, she developed from an egg that was fertilized outside her mother's body - the process called **in vitro fertilization** (**IVF**). IVF involves

- Harvesting mature eggs from the mother. This is not an easy process. The mother must undergo hormonal treatments to produce multiple eggs, which then must be removed (under anesthesia) from her ovaries.
- Harvesting sperm from the father. Harvesting is usually no problem, but often the sperm are defective in their ability to fertilize (so setting the stage for ICSI).
- Mixing sperm and eggs in a culture vessel ("in vitro").
- Culturing the fertilized eggs for several days until they have developed to at least the 8-cell stage.
- Placing two or more of these into the mother's uterus (which her hormone treatments have prepared for implantation).
- Keeping one's fingers crossed as only about one-third of the attempts result in a successful pregnancy.

Intra cytoplasmic Sperm Injection (ICSI)

Successful IVF assumes the availability of healthy sperm. But many cases of infertility arise from defects in the father's sperm. Often these can be overcome by directly injecting a single sperm into the egg. In the U.S. today, some two-thirds of ART procedures employ ICSI (even though as many as half of these do not involve male infertility).

Ooplasmic Transfer

Infertility in some cases may stem from defects in the cytoplasm of the mother's egg. To circumvent these, cytoplasm can be removed from the egg of a young, healthy woman ("Donor egg") and injected along with a single sperm into the prospective mother's egg. Although a few healthy children have been born following ooplasmic transfer, the jury is still out on its safety, and it is not approved for use in the U.S. One reason for concern is that ooplasmic transfer results in an egg carrying both the mother's mitochondria and mitochondria from the donor (in normal fertilization, all the mitochondria in the father's sperm are destroyed in the egg). This condition called **heteroplasmy** creates a child having two different mitochondrial DNA genomes in all of its cells.

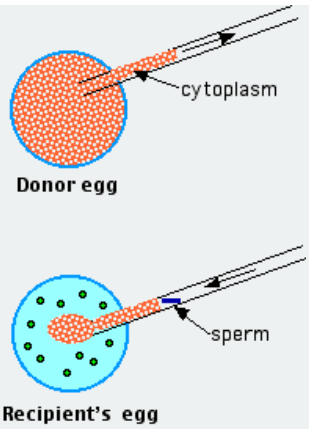


Fig.15.7.1.9 ooplasmic transfer

In rare, but important, cases, the defect in the prospective mother's cytoplasm is the result of her having mitochondria with a mutant gene. Ooplasmic transfer is of no help in these cases because the fertilized egg will still contain a preponderance of the mother's defective mitochondria. But three techniques worked out on laboratory animals show promise of being adapted to aid such women to produce healthy young.

Three Possible Ways to Prevent Transmission of Mitochondrial Diseases

Maternal Spindle Transfer

Researchers in Oregon reported in the 17 September 2009 issue of **Nature** that they had been able to produce 4 healthy rhesus monkeys with no mitochondria from their biological mother.

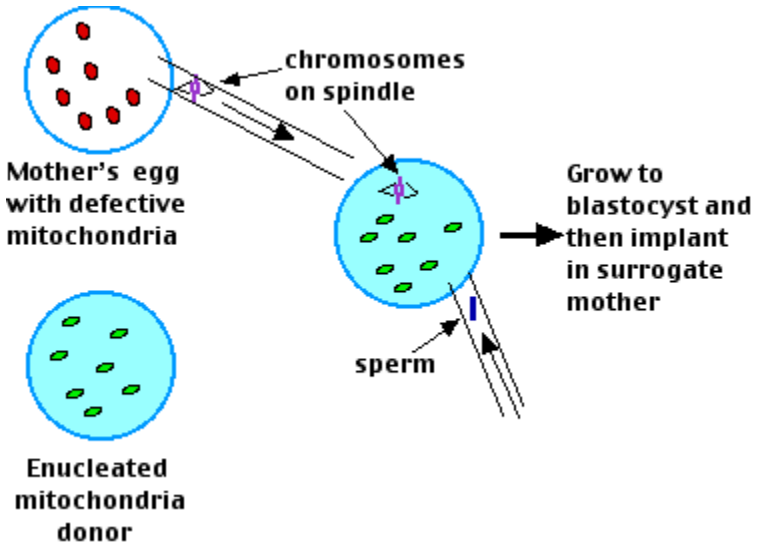


Figure 15.7.1.10 Maternal spindle transfer

Their procedure:

- Remove the spindle with all its attached chromosomes from the mother's oocyte at metaphase II of meiosis. They managed to do this without any of her mitochondria being withdrawn as well.
- Enucleate the oocyte of the mitochondria donor and then insert the mother's chromosomes — still attached to the spindle — into it. Then inject a sperm from the father.
- Allow the fertilized egg to develop into a blastocyst.
- Implant this in the uterus of a surrogate mother.

- The result: 4 healthy babies each with the nuclear genes of their mother and father but none of the mitochondria of their mother.

Pronuclear Transfer

In this procedure,

- An egg containing mutant mitochondria is fertilized by IVF.
- The two pronuclei are removed and injected into a fertilized egg with healthy mitochondria and whose own pronuclei have been removed.
- Allow the egg to develop into a blastocyst.
- Implant this in the uterus.

Both procedures (1) and (2) are under investigation for use in humans.

Polar Body Transfer

Both the first polar body (formed before fertilization) and the second polar body (formed after fertilization) contain a genome equivalent to that of the secondary oocyte and zygote respectively. However, they contain few, if any, mitochondria. In mice, transfer of either polar body to an enucleated recipient egg (with healthy mitochondria) yield young mice with few, if any, of the donor mother's mitochondria with their defective mtDNA. So in mice, at least, this technique produces offspring with less heteroplasmy than the other two techniques.

If any of these techniques can be applied to humans (there are safety and regulatory hurdles still to be overcome), it would allow women carrying defective mitochondria to bear babies free of the ailment.

The Upside of ART

- It has allowed some four million previously-infertile couples to have children.
- It permits screening (on one cell removed from the 8-celled morula) for the presence of genetic disorders - thus avoiding starting a pregnancy if a disorder is found.
- One can use frozen sperm allowing fatherhood for a man who is no longer able to provide fresh sperm.
- Because a number of morulas are created, the extras can be frozen, stored, and used later
    - if the initial attempt fails (the prospective mother must still receive hormones to prepare her uterus for implantation and the success rate is lower with thawed morulas).
    - Where regulations permit, the extras can be used as a source of embryonic stem (ES) cells.

The Downside of ART

- Although improving, the success rate is still sufficiently low (~35%) that the process often has to be repeated (which is physically demanding as well as very expensive).
- Because several morulas are usually transferred, multiple births are common (about 50%), and as is often the case with multiple births, the babies are born early and weigh less (~one-third of all ART babies in the U.S. are born early). To reduce the number of twins, triplets, etc., more ART centers are turning to **"single-embryo transfer"** (SET). Some ART centers find that they can increase the success rate and thus rely more on SET by culturing the morulas for 5–6 days, instead of the usual 2–3 days, before transferring them (by now they have become blastocysts) to the mother.
- The risk of birth defects may be increased slightly (from ~6% in "normal" pregnancies to ~8% in ART pregnancies).
- ART procedures in experimental animals often result in a failure of correct gene imprinting. Whether this will pose a problem for humans remains to be seen.

Birth and Lactation

Exactly what brings about the onset of labor is still not completely understood. Probably a variety of integrated hormonal controls are at work. A growing body of evidence implicates a rise in the level of fetal DNA in the mother's blood as a trigger for the onset of labor. The first result of labor is the opening of the cervix. With continued powerful contractions, the amnion ruptures and the amniotic fluid (the "waters") flows out through the vagina. The baby follows, and its umbilical cord can be cut. The infant's lungs

expand, and it begins breathing. This requires a major switchover in the circulatory system. Blood flow through the umbilical cord, ductus arteriosus, and foramen ovale ceases, and the adult pattern of blood flow through the heart, aorta, and pulmonary arteries begins. In some infants, the switchover is incomplete, and blood flow through the pulmonary arteries is inadequate. Failure to synthesize enough nitric oxide (NO) is one cause. Shortly after the baby, the placenta and the remains of the umbilical cord (the "afterbirth") are expelled.

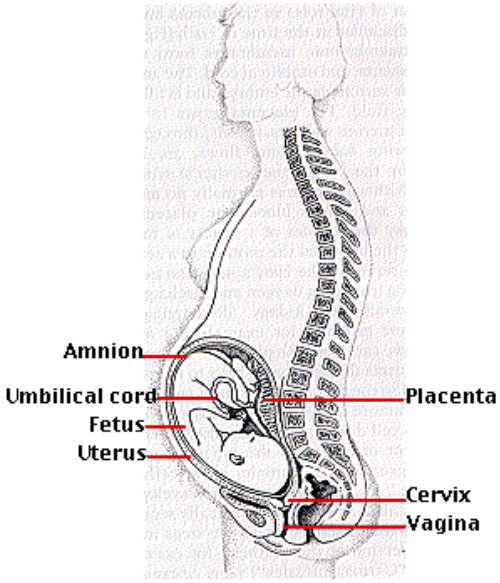


Figure 15.7.1.11 Fetus at full term

At the time of birth, and for a few days after, the mother's breasts contain a fluid called **colostrum**. It is rich in calories and proteins, including antibodies that provide passive immunity for the newborn infant. Three or four days after delivery, the breasts begin to secrete milk.

- Its synthesis is stimulated by the pituitary hormone **prolactin (PRL)**.
- Its release is stimulated by a rise in the level of **oxytocin** when the baby begins nursing.
- Milk also contains an **inhibitory peptide**. If the breasts are not fully emptied, the peptide accumulates and inhibits milk production. This autocrine action thus matches supply with demand.

Birth Control

The following table summarizes the various birth control method available and in use today.

Popularity (% using the method) and relative effectiveness of several methods of birth control among U. S. women using contraceptives. The pregnancy rate is the number of pregnancies per 100 women in the first year of using the method.

Method	Popularity	Pregnancy Rate
Natural family planning (rhythm)	1%	25
Male condom	16%	17
Oral contraceptives ("the pill")	28%	0.3–8.7*
Intrauterine devices (IUD)	8.5%	0.1–1.0*
Implants, e.g., Implanon®	~1%	0.05–1.0*
DMPA injections	~3.5%	6.7
Male + Female Sterilization	37%	<<1%
None		85
* The lower value is found under ideal conditions; i.e., among highly-motivated women receiving good medical care.		

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15.7B: Asexual Reproduction in Animals

Asexual reproduction is the formation of new individuals from the cell(s) of a single parent. It is very common in plants; less so in animals.

Asexual Reproduction in Plants

All plant organs have been used for asexual reproduction, but stems are the most common.

Stems



Figure 15.7.2.1 Stolons of strawberry plant

In some species, stems arch over and take root at their tips, forming new plants. The horizontal above-ground stems (called **stolons**) of the strawberry produce new daughter plants at alternate nodes. Underground stems such as rhizomes, bulbs, corms and tubers are used for asexual reproduction as well as for food storage. Irises and day lilies, for example, spread rapidly by the growth of their rhizomes.

Leaves



Figure 15.7.2.2 Leaves of bryophyllum

The above photo shows the leaves of the common ornamental plant Bryophyllum (also called Kalanchoë). Mitosis at meristems along the leaf margins produce tiny plantlets that fall off and can take up an independent existence.

Roots

Some plants use their roots for asexual reproduction. The dandelion is a common example. Trees, such as the poplar or aspen, send up new stems from their roots. In time, an entire grove of trees may form - all part of a **clone** of the original tree.

Plant Propagation

Commercially-important plants are often deliberately propagated by asexual means in order to keep particularly desirable traits (e.g., flower color, flavor, resistance to disease). Cuttings may be taken from the parent and rooted.

Grafting

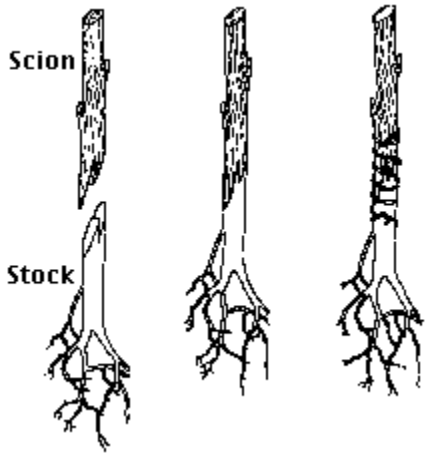


Figure 15.7.2.3 Grafting

Grafting is widely used to propagate a desired variety of shrub or tree. All apple varieties, for example, are propagated this way.

Apple **seeds** are planted only for the root and stem system that grows from them. After a year's growth, most of the stem is removed and a twig (**scion**) taken from a mature plant of the desired variety is inserted in a notch in the cut stump (the **stock**). So long the cambiums of scion and stock are united and precautions are taken to prevent infection and drying out, the scion will grow. It will get all its water and minerals from the root system of the stock. However, the fruit that it will eventually produce with be identical (assuming that it is raised under similar environmental conditions) to the fruit of the tree from which the scion was taken.

Apomixis

Citrus trees and many other species of angiosperms use their seeds as a method of asexual reproduction; a process called **apomixis**.

- In one form, the egg is formed with **2n** chromosomes and develops without ever being fertilized.
- In another version, the cells of the ovule (**2n**) develop into an embryo instead of - or in addition to - the fertilized egg.

Hybridization between different species often yields infertile offspring. But in plants, this does not necessarily doom the offspring. Many such hybrids use apomixis to propagate themselves. The many races of Kentucky bluegrass growing in lawns across North America and the many races of blackberries are two examples of sterile hybrids that propagate successfully by apomixis. Recently, an example of apomixis in gymnosperms was discovered (see Pichot, C., et al, in the 5 July 2001 issue of **Nature**). In a rare cypress, the pollen grains are diploid, not haploid, and can develop into an embryo when they land on either

- the female cones of their own species (rare) or
- those of a much more common species of cypress.

Is this **paternal apomixis** in a surrogate mother a desperate attempt to avoid extinction?

Breeding apomictic crop plants

Many valuable crop plants (e.g., corn) cannot be propagated by asexual methods like grafting.

Agricultural scientists would dearly love to convert these plants to apomixis: making embryos that are genetic clones of

themselves rather than the product of sexual reproduction with its inevitable gene reshuffling. After 20 years of work, an apomictic corn (maize) has been produced, but it does not yet produce enough viable kernels to be useful commercially.

Asexual Reproduction in Animals

Budding

Here, offspring develop as a growth on the body of the parent. In some species, e.g., jellyfishes and many echinoderms, the buds break away and take up an independent existence. In others, e.g., corals, the buds remain attached to the parent and the process results in **colonies** of animals. Budding is also common among parasitic animals, e.g., tapeworms.

Fragmentation

As certain tiny worms grow to full size, they spontaneously break up into 8 or 9 pieces. Each of these fragments develops into a mature worm, and the process is repeated.

Parthenogenesis

In parthenogenesis ("virgin birth"), the females produce eggs, but these develop into young without ever being fertilized. Parthenogenesis occurs in some fishes, several kinds of insects, and a few species of frogs and lizards. It does not normally occur in mammals because of their imprinted genes. However, using special manipulations to circumvent imprinting, laboratory mice have been produced by parthenogenesis.

In a few nonmammalian species it is the only method of reproduction, but more commonly animals turn to parthenogenesis only under certain circumstances. Examples:

- Aphids use parthenogenesis in the spring when they find themselves with ample food. In this species, reproduction by parthenogenesis is more rapid than sexual reproduction, and the use of this mode of asexual reproduction permits the animals to quickly exploit the available resources.
- Female Komodo dragons (the largest lizard) can produce offspring by parthenogenesis when no male is available for sexual reproduction. Their offspring are homozygous at every locus including having identical sex chromosomes. Thus the females produce all males because, unlike mammals, females are the heterogametic sex (ZW) while males are homogametic (ZZ).

Parthenogenesis is forced on some species of wasps when they become infected with bacteria (in the genus **Wolbachia**). Wolbachia can pass to a new generation through eggs, but not through sperm, so it is advantageous to the bacterium for females to be made rather that males. In these wasps (as in honeybees), fertilized eggs (diploid) become females and unfertilized (haploid) eggs become males. However, in Wolbachia-infected females, all their eggs undergo *endoreplication* producing diploid eggs that develop into females without fertilization, by parthenogenesis. Treating the wasps with an antibiotic kills off the bacteria and "cures" the parthenogenesis!

Apis mellifera capensis

Occasionally worker honeybees develop ovaries and lay unfertilized eggs. Usually these are haploid, as you would expect, and develop into males. However, workers of the subspecies ***Apis mellifera capensis*** (the Cape honeybee) can lay unfertilized **diploid** eggs that develop into females (who continue the practice). The eggs are produced by meiosis, but then the polar body nucleus fuses with the egg nucleus restoring diploidy (2n). The phenomenon is called **automictic thelytoky**.



Cape honey bees gorging on honey. (CC BY-SA 3.0; Discott)

Why Choose Asexual Reproduction?

Perhaps the better question is: **Why not?** After all, asexual reproduction would seem a more efficient way to reproduce. Sexual reproduction requires males but they themselves do not produce offspring. Two general explanations for the overwhelming prevalence of sexually-reproducing species over asexual ones are:

- Perhaps sexual reproduction has kept in style because it provides a mechanism to weed out (through the recombination process of meiosis) harmful mutations that arise in the population reducing its fitness. Asexual reproduction leads to these mutations becoming homozygous and thus fully exposed to the pressures of natural selection.
- Perhaps it is the ability to adapt quickly to a changing environment that has caused sex to remain the method of choice for most living things.

Purging Harmful Mutations

Most mutations are harmful - changing a functional allele to a less or nonfunctional one. An asexual population tends to be genetically static. Mutant alleles appear but remain forever associated with the particular alleles present in the rest of that genome. Even a beneficial mutation will be doomed to extinction if trapped along with genes that reduce the fitness of that population. But with the **genetic recombination** provided by sex, new alleles can be shuffled into different combinations with all the other alleles available to the genome of that species. A beneficial mutation that first appears alongside harmful alleles can, with recombination, soon find itself in more fit genomes that will enable it to spread through a sexual population.

Evidence (from Paland and Lynch in the 17 February 2006 issue of **Science**): Some strains of the water flea ***Daphnia pulex*** (a tiny crustacean) reproduce sexually, others asexually. The asexual strains accumulate deleterious mutations in their mitochondrial genes four times as fast as the sexual strains.

Evidence (from Goddard *et al*. in the 31 March 2005 issue of **Nature**): Budding yeast missing two genes essential for meiosis adapt less rapidly to growth under harsh conditions than an otherwise identical strain that can undergo genetic recombination. Under good conditions, both strains grow equally well.

Evidence (from Rice and Chippindale in the 19 October 2001 issue of **Science**):

Using experimental Drosophila populations, they found that a beneficial mutation introduced into chromosomes that **can** recombine did over time increase in frequency more rapidly than the same mutation introduced into chromosomes that could **not** recombine.

So sex provides a mechanism for testing **new combinations of alleles** for their possible usefulness to the phenotype:

- deleterious alleles weeded out by natural selection
- useful ones retained by natural selection

Some organisms may still gain the benefits of genetic recombination while avoiding sex. Many mycorrhizal fungi use asexual

reproduction only. However, at least two species have been shown to have multiple — similar — copies of the same gene; that is, are polyploid. Perhaps recombination between these (during mitosis?) enables these organisms to avoid the hazards of accumulating deleterious mutations. (See the paper by Pawlowska and Taylor in the 19 Feb 2004 issue of **Nature**.) But there are many examples of populations that thrive without sex, at least while they live in a **stable environment**.

Red Queen hypothesisnment

As we have seen (above), populations without sex are genetically static. They may be well-adapted to a given environment, but will be handicapped in evolving in response to changes in the environment. One of the most potent environmental forces acting on a species environment is its parasites. The speed with which parasites like bacteria and viruses can change their virulence may provide the strongest need for their hosts to have the ability to make new gene combinations. So sex may be virtually universal because of the never-ending need to keep up with changes in parasites.

Evidence:

- Some parasites interfere with sexual reproduction in their host:
  - Wolbachia-induced parthenogenesis discussed above is an example.
  - Several types of fungi block wind pollination of their grass hosts forcing them to inbreed with its resulting genetic uniformity.
- There is some evidence that genetically uniform populations are at increased risk of devastating epidemics and population crashes.
- Flour beetles (*Tribolium castaneum*) parasitized by the microsporidium *Nosema whitei* increase the rate of recombination during meiosis.
- *Drosophila* females parasitized by bacteria produce more recombinant offspring than non-infected mothers do.

The idea that a constantly-changing environment, especially with respect to parasites, drives evolution is often called the **Red Queen** hypothesis. It comes from Lewis Carroll's book *Through the Looking Glass*, where the Red Queen says "Now here, you see, it takes all the running you can do to keep in the same place".

The possibilities outlined above are not mutually exclusive and a recent study [see Morran, L. T., *et al*., in *Nature*, **462**:350, 19 November 2009] suggests that both forces are at work in favoring sexual reproduction over its alternatives.

The organism for testing these theories was ***Caenorhabditis elegans***. While *C. elegans* does not reproduce asexually, most worms are hermaphrodites and usually reproduce by self-fertilization with each individual fertilizing its own eggs. This quickly results in its genes becoming homozygous and thus fully-exposed to natural selection just as they are in asexually-reproducing species. Hermaphrodites have two X chromosomes and self-fertilization ("selfing") usually produces more of the same; that is, hermaphrodites produce more hermaphrodites. However, an occasional nondisjunction generates an embryo with a single X chromosome and this develops into a male. These males can mate with hermaphrodites (their sperm is preferred over the hermaphrodites own) and, in fact, such "outcrossing" produces a larger number of offspring. It also produces 50% hermaphrodites and 50% males.

Testing the role of outcrossing vs. self-fertilization in maintaining fitness in the face of an increased mutation rate

These workers developed six strains of worms:

- two that could reproduce only by selfing
- two that could reproduce only by crossing a male with an hermaphrodite ("outcrossing")
- "wild-type" worms

All the strains were exposed to a chemical mutagen that increased the spontaneous mutation rate some fourfold.

The results: After 50 generations, the

- the strains of worms that could reproduce only by selfing suffered a serious decline in fitness
- the strains of worms that could reproduce only by outcrossing suffered no decline
- the wild-type worms with intermediate levels of outcrossing (20–30%) suffered only moderate declines in fitness

Fitness was measured by placing the worms in a petri dish with a barrier that they had to cross to reach their food (*E. coli*).

The conclusion: the genetic recombination provided by outcrossing protected the worms from loss of fitness even in the face of an increase in mutation rate.

Testing the role of outcrossing vs. self-fertilization in the speed of adaptation to a changed environment

For these tests, one of each category of mating types was exposed over 40 generations to a pathogenic bacterium (*Serratia marcescens*) that killed most worms when eaten by them.

The results: After 40 generations,

- the strain of worms that could reproduce only by selfing were just as susceptible to the pathogen as they were at the start while
- the strain of worms that could reproduce only by outcrossing had evolved a high degree of resistance to the pathogen
- the wild-type worms only developed a modest increase in their resistance to the bacteria.

Since these studies were reported, the same team has expanded their experiments to examine the effects of evolution in the pathogen (*Serratia marcescens*), that is, to look for evidence of **coevolution** of host and parasite. (Reported by Morran, L. T., *et al*., in *Science*, **333**: 216, 8 July 2011.)

Over 30 generations of worms, they harvested and tested the bacteria recovered from the bodies of worms that had died within 24 hours of infection. They found that:

- Worms that can maintain genetic variability by outcrossing suffered substantially lower mortality from the coevolved parasite that did worms from the starting population (kept frozen until used).
- Worms that could only reproduce by selfing became so susceptible to the evolving strain of *Serratia marcescens* that they died out within 20 generations.
- Curiously, the selection pressure of the increasing virulence of *Serratia marcescens* caused wild-type worms to increase their rate of outcrossing from the normal 20–30% to over 80%. So one response to the pressure of this coevolving parasite was to promote sex in its host.

Reproduction in Rotifers

Rotifers are microscopic invertebrates. They are assigned a phylum of their own (not discussed elsewhere in these pages). The phylum includes:

- a class of ~1,500 species called monogonont rotifers (they have only a single gonad). The monogonont rotifers can choose either asexual or sexual reproduction as circumstances warrant.
- a class of ~350 species called bdelloid rotifers. The bdelloid rotifers are limited to asexual reproduction only. Even after years of study, neither males nor haploid eggs have ever been found in any members of this group. It looks as though they gave up sexual reproduction millions of years ago.



A bdelloid rotifer. (CC BY-SA 3.0; Bob Blaylock)

Laboratory studies show that monogonont rotifers favor asexual reproduction when they are living in a stable environment but shift to more sexual reproduction when placed in a varied or unfavorable environment. As they adapt to the new environment, they gradually switch back to asexual reproduction.

But how have the bdelloid rotifers that never engage in sexual reproduction managed to survive? How have they avoided the demands of the *Red Queen*; that is, avoided extinction at the hands of parasites?

One study (Wilson, C. G. and Sherman, P. W., **Science**, **327**:574, 29 January 2010) reveals a mechanism. These tiny animals can be completely desiccated (dried out) and remain in suspended animation for years. In the desiccated state, they can be blown vast distances (some species are worldwide in their distribution). Once deposited in a moist environment (a few drops of water are sufficient), they resume an active life. Wilson and Sherman have shown that the desiccation that is harmless to the rotifers is lethal to their fungal parasite. So once dried, they are not only cured of their parasite, but can then be blown to a spot where they can resume an active life with no parasites present.

Another way in which these rotifers can avoid the evolutionary dead-end expected of asexually-reproducing organisms has been revealed by DNA sequencing of their genome. It turns out that they can purge their genome of deleterious alleles by gene conversion (during mitosis).

In any case despite its disadvantages sexual reproduction is here to stay

- reducing the effect of harmful mutations
- increasing the speed with which populations can adapt to changes in their environment

Contibutors

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15.7C: Birth Control

Mechanical and/or Chemical Barriers

Male Condom

- a sheath of thin, flexible material (e.g., latex) worn over the penis
- effective if used carefully
- also protects against sexually-transmitted disease (STD) agents such as
  - HIV, the cause of AIDs
  - herpes virus
  - human papilloma virus (**HPV**)
  - chlamydiae
  - *Neisseria gonorrhoeae*, the cause of gonorrhea

Female Condom

- a thin-walled pouch inserted into the vagina
- protects against sexually-transmitted diseases (STDs)
- less effective than the male condom

Diaphragm

- rubber dome placed at the upper end of the vagina
- may be used along with spermicides

Cervical Cap

- impermeable cap fitted over the cervix
- best used with spermicides

Spermicides

- chemicals, such as nonoxynol 9, that inactivate sperm. Inserted into the vagina — often incorporated in a sponge — prior to intercourse.
- not very effective if used alone

Hormonal Contraception

Oral Contraceptives - the "Pill"

- Many formulations combining varying amounts of a **synthetic estrogen** and a synthetic **progestin** (progesterone-like steroid)
- taken for 3 weeks, then stopped to allow menstruation
- most widely-used reversible method

Skin ("Transdermal") Patch

- The Ortho Evra® patch releases an estrogen and a progestin through the skin.
- A fresh patch is applied each week for 3 weeks, and then a week without allows menstruation.

- The failure rate is about 9%.

Vaginal Ring

- a small plastic ring inserted into the vagina
- NuvaRing® releases both an estrogen and a progestin.
- It is left in place for 3 weeks and then removed for a week to allow menstruation.
- The failure rate is about 9%.

Injectable Contraceptive

- An injection containing a synthetic progestin (**d**epot **m**edroxy**p**rogesterone **a**cetate or "DMPA")(Depo-Provera®)
- One injection given every three months inhibits the release of GnRH, thus inhibiting the synthesis of FSH and LH and blocking ovulation.
- One of the most reliable methods of birth control.

Contraceptive Implant

- Implanon® and Nexplanon®, each a tiny (40 x 2 mm) flexible plastic rod that releases a synthetic progestin is inserted under the skin (requiring a local anesthetic).
- Prevents pregnancy for up to 3 years.
- If pregnancy is desired sooner, is easily removed (again requiring a small incision and a local anesthetic) and normal fertility quickly returns.
- Although used by only ~1% of U.S. women, implants are among the most effective of the birth control methods.

"Morning After" Pill

The most popular formulation in the U.S., called **Plan B One-Step®**, contains a high dose of a progestin. If taken within 72 hours after unprotected intercourse, the drug interferes with ovulation and, if ovulation has occurred, with fertilization.

If so many days have elapsed that implantation has occurred, RU-486 may be used. **RU-486** is a synthetic steroid related to progesterone. Unlike the progestins discussed above, that mimic the action of progesterone, RU-486 blocks the action of progesterone. (Synthetic molecules that mimic the action of a natural molecule are called **agonists**; those that oppose it are **antagonists**.) RU-486 (also known as mifepristone) is a progesterone **antagonist**. It binds to the progesterone receptor, and in so doing prevents progesterone itself from occupying its receptor. Thus the gene transcription normally turned on by progesterone is blocked, and the proteins necessary to begin and maintain pregnancy are not synthesized. Because RU-486 is used after implantation, it is causing an early abortion and thus has been subjected to controversy.

Intrauterine Devices (IUD)

- The intrauterine device (IUD) is a device, usually T-shaped, inserted into the uterus by a physician.
- Two types available in the U.S.:
  - ParaGard®, which is coated with copper wire and can be left in place for 10 years;
  - Mirena®, which releases a progestin and can be left in place for up to 5 years.
- Although used by only 8.5% of U.S. women, IUDs are among the most effective of the birth control methods. Only about 1 woman in a thousand becomes pregnant during her first year of using Mirena®.

Natural Family Planning - Rhythm Methods

- An egg must be fertilized on the day of ovulation.
- Sperm can live in the female reproductive tract for up to 6 days.
- So copulation that takes place more than 5 days before or a day after ovulation is unlikely to lead to pregnancy.
- Abstinence during this period is called natural family planning or the rhythm method.
- Its success (which is low) depends upon being able to determine accurately just when ovulation occurs.

- Highly-motivated women can do this by
  - monitoring their body temperature (which rises slightly at ovulation)
  - the amount and consistency of the mucus secreted by their uterus, and more recently
  - measuring the concentration of estrogen and/or progesterone in the urine (which mirrors the level in the blood).
- It is favored by those who do not currently want a baby, but do not wish to use contraceptive devices (about 1% of U.S. couples).

Abortion

- the deliberate removal of the embryo or fetus before it is ready for birth
- Done
  - mechanically
    - using a suction device (during the first 3 months of pregnancy)
    - using surgery (later in pregnancy)

    or
  - chemically (using RU-486 and prostaglandins) during the first 7 weeks of pregnancy

All methods of birth control have been the subject of controversy (except for natural family planning).

- In general, the controversy over a given method is proportional to the lateness of the stage of the reproductive process.
- So not surprisingly abortion is a particularly controversial procedure especially when it is induced in the later stages of pregnancy.
- Nevertheless, worldwide some 46 million pregnancies are terminated each year by induced abortion.

Sterilization

Roughly one-third of U.S. couples still in their reproductive years have chosen for one or the other to be sterilized.

Tubal Ligation

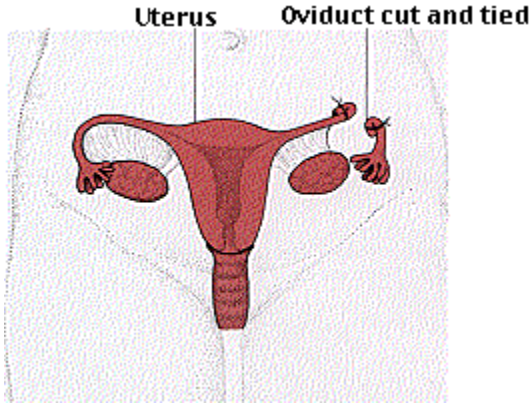


Figure 15.7.3.1 Tubal ligation

- Both fallopian tubes (oviducts) are cut and tied so that no egg can be fertilized.
- Requires incision(s) and so must be done under anesthesia.

Vasectomy

- Each vas deferens is cut near the top of the scrotum.
- Can be done in the doctor's office, with a local anesthetic, in 30-40 minutes.
- Testosterone secretion by the testes is not inhibited.
- Does not stop the production of the various glandular secretions that make up the bulk of the semen.
- Copulation and ejaculation proceed normally.
- Sometimes the operation can be reversed, but don't count on it.

Quinacrine Sterilization (QS)

- Pellets of the antimalarial drug quinicrine are placed (by a physician) in the uterus
- Done twice, a month apart.
- Causes scarring of the fallopian tubes
- Clinical trials are in progress in the U.S.

Summary

Popularity (% using the method) and relative effectiveness of several methods of birth control among U. S. women using contraceptives. The pregnancy rate is the number of pregnancies per 100 women in the first year of using the method.

Method	Popularity	Pregnancy Rate
Natural family planning (rhythm)	1%	25
Male condom	16%	17
Oral contraceptives ("the pill")	28%	0.3–8.7*
Intrauterine devices (IUD)	8.5%	0.1–1.0*
Implants, e.g., Implanon®	~1%	0.05–1.0*
DMPA injections	~3.5%	6.7
Male + Female Sterilization	37%	<<1%
None		85

* The lower value is found under ideal conditions; i.e., among highly-motivated women receiving good medical care.

The bottom line: The failure rate of the pill, patch, and vaginal ring, as they are commonly used, is 20 times that in women using an IUD, or implant.

Future Prospects

- Not too bright because pharmaceutical houses are reluctant to invest the huge amounts of money and time needed to develop products that expose them to controversy, put them at risk of lawsuits and whose largest market is in countries too poor to afford them.
- Research on possible male birth control pills is going on. However, it is not yet easy to see how spermatogenesis can be blocked without causing undesirable side-effects.
- Some research is proceeding on contraceptive **vaccines**; that is, using the immune system to block one or another step in the process (e.g. fertilization). Examples: vaccines to raise antibodies
  - against gonadotropin-releasing hormone, **GnRH** (for males)
  - against human chorionic gonadotropin, **HCG** (for females)
  - to immobilize sperm (also for females)

  But, what risks such antibodies might present is not at all clear.

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15.7D: Prenatal Screening

Many tests are now available to detect genetic diseases such as sickle cell disease, cystic fibrosis and phenylketonuria (PKU). Most of these tests can not only be performed on cells removed from adults but also on cells removed from the fetus and even from a pre-implantation embryo.

Amniocentesis

During its development, the fetus sheds cells into the amniotic fluid. After 14–22 weeks of pregnancy, a small volume of this fluid can be removed (using a needle inserted through the abdominal wall).

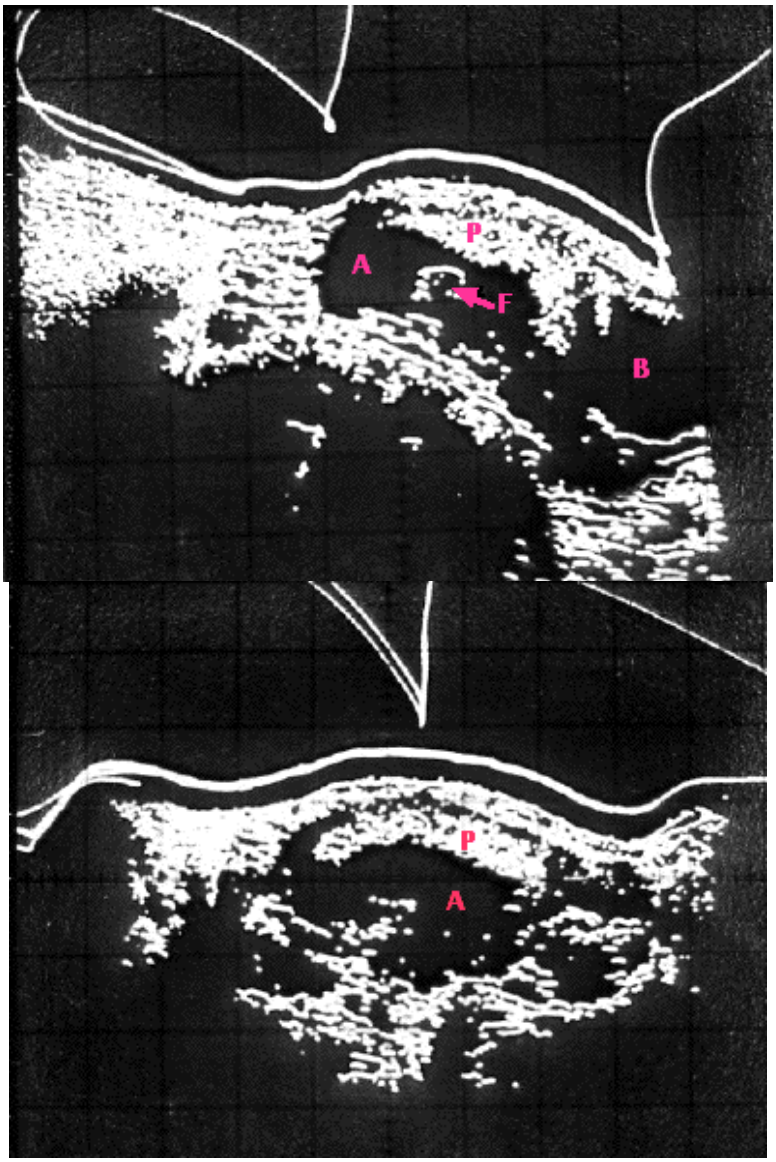


Figure 15.7.4.1 Ultrasound prior to amniocentesis

Using ultrasound to locate the position of the placenta prior to amniocentesis. These sonograms are made by recording the echoes received from structures within the abdomen. A, amniotic cavity; B, urinary bladder; F, part of the fetus; P, placenta. Both longitudinal (left) and transverse (right) scans are needed for accurate localization of the placenta. (Courtesy of the Downstate Medical Center of the State University of New York.)

Separating the cells and culturing them enables the clinician to look for

- chromosome abnormalities (e.g., the three number 21 chromosomes of Down syndrome)
- certain enzymatic defects (e.g., an inability to metabolize galactose, hence milk)
- the sex of the fetus

Over 100 genetic abnormalities can be diagnosed by amniocentesis and the pregnancy deliberately ended if the parents wish it.

Chorionic Villus Sampling (CVS)

This is an alternate method of prenatal diagnosis. A small amount of placental tissue is sucked out by a tube inserted through the abdominal wall or through the vagina (the latter avoiding the need for an incision). For some tests the fetal cells can be examined immediately without the need to culture them. Another advantage of CVS is that it can be performed earlier in pregnancy (after only 10–12 weeks) than amniocentesis. If an abortion is to be performed, it is a simpler process early in pregnancy.

Non-Invasive Prenatal Genetic Testing (NIPT)

Although the blood vessels of the placenta are in close contact with the mother's blood vessels in the uterus, intermingling of their blood does not normally occur. However some of cells of the fetus do manage to get into the mother's circulation where they may represent 1 in a million of her white blood cells (so only some 2–6 cells per ml of blood). Fragments of fetal DNA (~ 300 bp long) from apoptotic cells of the placenta are also found in the mother's plasma as early as 5 weeks after implantation. This raises the possibility of using genetic tests (e.g., PCR) to identify mutations or chromosomal abnormalities in the fetus using a small (~10 ml) sample of blood drawn from the mother.

Two home blood test kits for determining the sex of the fetus are already on the market. The collected drops of blood are sent to a laboratory to determine whether any Y-chromosome-specific DNA (e.g., *SRY*) is present. Tests of fetal DNA for Down syndrome (trisomy 21), as well as for trisomy 13 and 18, have both higher sensitivity (false negatives <0.1%) and specificity (false positives <0.2%) than amniocentesis and CVS.

In several European countries, Rh-negative mothers can now have their blood screened for the presence of an Rh-positive fetus. The time will surely come when such NIPT screening will become available for many genetic disorders. The procedure is also called non-invasive prenatal diagnosis - NIPD.

The level of fetal DNA in the mother's blood rises to a peak at the time of birth and some evidence suggests that this rise may be a trigger to start the birth process.

Preimplantation Genetic Diagnosis (PGD)

Genetic Analysis of Blastomeres

One of the remarkable facts about mammalian development is that all the cells in the early (e.g., 8-cell) embryo are not needed to produce a healthy fetus (which is why a single fertilized egg can on occasions produce identical twins, triplets, etc.). So couples using in vitro fertilization (IVF) also can take advantage of genetic screening. While the embryo is in culture, one or two cells can safely be removed and tested for their genotype. For example:

- The sex of the embryo can be determined with a probe for Y-specific DNA. This permits prospective mothers carrying a severe X-linked trait like hemophilia A to choose a female rather than a male embryo for attempted implantation.
- Fluorescent probes specific for the DNA of particular chromosomes can detect (by FISH) if there is an abnormal number (aneuploidy) such as the three #21 chromosomes of Down syndrome.
- In fact the entire karyotype of the embryo can be determined. Random fragments of DNA prepared by the polymerase chain reaction (PCR) of all the DNA of a cell from the **embryo** can be
  - given a fluorescent label
  - applied to the metaphase **chromosomes** of a standard reference cell that has a normal karyotype along with
  - DNA fragments from the reference cell labelled with a different color.

  Comparing the intensity of the two colors from each chromosome shows whether the embryo has the normal amount of DNA for that chromosome or is aneuploid containing either:

- too much (e.g. 3 copies of #21 — trisomy)
- too little (only a single copy of #14 — monosomy)

Genetic Analysis of Polar Bodies

As meiosis I is completed, one set of duplicated chromosomes (dyads) is extruded into the first polar body. The DNA of the polar body can be amplified by the polymerase chain reaction (PCR) and tested.

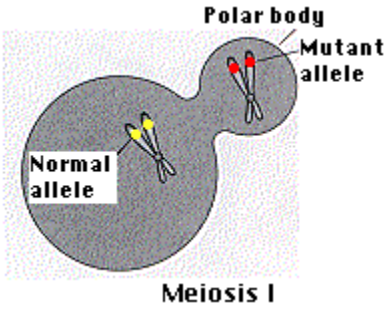


Figure 15.7.4.2 Polar body screening

If the mother is heterozygous for a trait, **and no crossover** has occurred, and the polar body contains the mutant alleles (see figure), the egg can be safely fertilized. (For simplicity, the figure shows only the pair of homologues carrying the locus of concern.)

However, if crossing over has occurred, the first polar body would contain one mutant and one healthy allele. In that case, there is a 50:50 chance that, after fertilization, the other copy of the mutant allele will end up in the egg (instead of in the second polar body). So the second polar body should also be tested to see if it also contains the mutant allele. Only if it does can the egg be safely used.

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15.7E: Extraembryonic Membranes and the Physiology of the Placenta

This page discusses embryo development in reptiles, birds, and mammals, focusing on four extraembryonic membranes: amnion, yolk sac, chorion, and allantois, which support the embryo. It categorizes mammals into monotremes, marsupials, and placental mammals, highlighting that humans belong to the placental group, where the placenta connects the embryo to the mother.

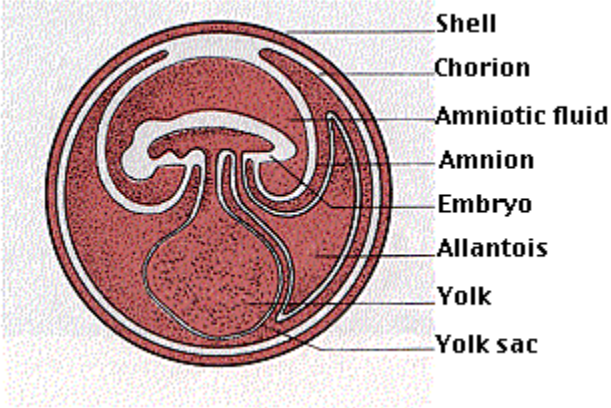


Figure 15.7.5.1 Amniotic egg

The embryos of reptiles, birds, and mammals produce 4 extraembryonic membranes - amnion, yolk sac, chorion and allantois. In birds and most reptiles, the embryo with its extraembryonic membranes develops within a shelled egg.

- The **amnion** protects the embryo in a sac filled with **amniotic fluid**.
- The **yolk sac** contains yolk — the sole source of food until hatching. Yolk is a mixture of proteins and lipoproteins.
- The **chorion** lines the inner surface of the shell (which is permeable to gases) and participates in the exchange of O_2 and CO_2 between the embryo and the outside air.
- The **allantois** stores metabolic wastes (chiefly uric acid) of the embryo and, as it grows larger, also participates in gas exchange.

With these four membranes, the developing embryo is able to carry on essential metabolism while sealed within the egg. Surrounded by amniotic fluid, the embryo is kept as moist as a fish embryo in a pond. Although (most) mammals do not make a shelled egg, they do also enclose their embryo in an amnion. For this reason, the reptiles, birds, and mammals are collectively referred to as the **amniota**.

Amniotic Egg groups in Mammals

Mammals fall into three groups that differ in the way they use the amniotic egg.

- These primitive mammals produce a shelled egg like their reptilian ancestors. Only four species exist today: three species of spiny anteater (echidna) and the duckbill platypus.
- Marsupials

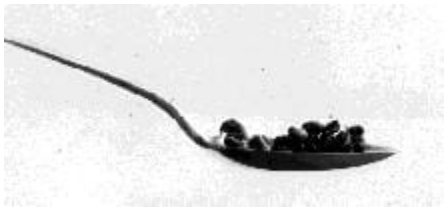


Figure 15.7.5.2 Newborn baby opossums courtesy of Dr. Carl G. Hartman

Marsupials do not produce a shelled egg. The egg, which is poorly supplied with yolk, is retained for a time within the

reproductive tract of the mother. The embryo penetrates the wall of the **uterus**. The yolk sac provides a rudimentary connection to the mother's blood supply from which it receives food, oxygen, and other essentials. However, this interface between the tissues of the uterus and the extraembryonic membranes never becomes elaborately developed, and the young are born in a very immature state.

The photo shows 18 newborn baby opossums fitting easily into a teaspoon. Despite their tiny size, they are able to crawl into a pouch on the mother's abdomen, attach themselves to nipples, and drink milk from her mammary glands. Marsupials are still abundant in Australia, but only the opossum is found in North America.

- **Placental mammals**
  In placental mammals, the extraembryonic membranes form a **placenta** and **umbilical cord**, which connect the embryo to the mother's uterus in a more elaborate and efficient way. The blood supply of the developing fetus is continuous with that of the placenta. The placenta extracts food and oxygen from the uterus. Carbon dioxide and other wastes (e.g., urea) are transferred to the mother for disposal by her excretory organs.

  Humans are placental mammals.

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15.7F: Genetic Mosaics

A genetic mosaic is a creature whose body is built of a **mixture** of cells of two or more different genotypes. In mammals they arise by several different mechanisms:

- The fusion of two different zygotes, or early embryos, into one. (The reverse of the process that produces identical twins!) The resulting animal is called a **chimera** (after the monster in Greek mythology with a lion's head, goat's body, and serpent's tail). The tetraparental mouse is a chimera formed this way. But on rare occasions, the same process can occur spontaneously in humans (especially those using in vitro fertilization).
- The sharing of blood supplies by separate embryos. This occurs with the occasional fraternal cattle twins and also — less often — with human **fraternal twins** who have shared the same placenta. Blood stem cells of each twin seed the bone marrow of the other. Only their blood cells are mosaic.
- During early development, errors during mitosis can produce stem cells that go on to populate a tissue or organ with, for example, a chromosomal aberration (e.g., aneuploid).

  Example: Occasionally a baby is born with blood cells that have three copies of chromosome 21 (the same set responsible for Down syndrome). This can produce a leukemia-like illness that, fortunately, often disappears as that cell population declines.

- All female mammals are mosaic for the genes on the X chromosome because of the random inactivation of one or the other X chromosome in all their somatic cells.
- Anyone unlucky enough to have a cancer is a genetic mosaic because all cancers are made up of the descendants of cells carrying a suite of mutations not found in normal cells.
- Recent advances have enabled the coding portions of the genome of **single cells** to be sequenced. Early results indicate than even normal cells in an adult have accumulated a suite of somatic mutations that differs from cell to cell. So all of us are genetic mosaics! However, the rate of somatic mutations in these normal cells is only a fourth of that in cancer cells.

The Tetraparental Mouse

As the name suggests, tetraparental mice have four parents: two fathers and two mothers (not including the foster mother that gives birth to them!). This is how they are made:

- Early embryos at the 8-cell stage are removed from two different pregnant mice and placed in tissue culture medium.
- Two different embryos are gently pushed together and, often, will fuse into a single embryo.
- After a period of further growth in culture, the fused embryo is implanted in a foster mother (whose uterus has been prepared for implantation by mating her with a vasectomized male).
- The mouse that is born is a **chimera**, all (usually) of whose organs are made of some cells derived from one pair of parents and some cells derived from the other pair.



Figure 15.7.6.1 Tetraparental mouse *courtesy of the late Dr. Thomas G. Wegmann*

The photograph shows a tetraparental mouse derived from a pair of inbred mice with black fur and a pair with white fur. Note the intermingling of black and white patches. This mouse is **not** the same as an F1 hybrid produced by mating a white mouse with a black one. In that case, all the cells would be of the same genotype, and the coat would have been a uniform brown.

A Tetragametic Human

A report by Yu, et. al. in the May 16, 2002 issue of **The New England Journal of Medicine** documents the discovery of a tetragametic woman; that is a woman derived from four different gametes, not just two. She came to the doctors' attention because she needed a kidney transplant.

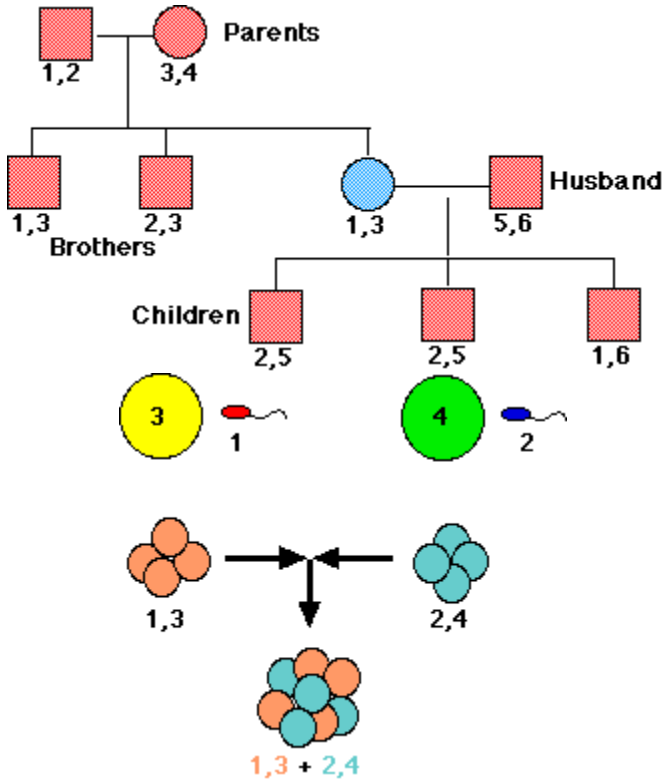


Figure 15.7.6.3 Chimera formation

- Tissue typing, which is done with **blood cells**, showed her to have inherited the "1" HLA region of her father (who was 1,2) and the "3" region of her mother (who was 3,4).
- She had two brothers,
- One who inherited 1 from their father and 3 from their mother
- The other who inherited 2 from their father and 3 from their mother.
- Her husband typed 5,6
- Of her three sons,
  - One was 1,6 which was to be expected
  - the other two were both 2,5. The 5 they got from their father, but where did the 2 come from?
- The first thought was that she could not have been their mother, but clearly she knew better. (Paternity may sometimes be in doubt, but not maternity.)
- A clue came from typing other tissues. DNA analysis of her skin cells, hair follicles, thyroid cells, bladder cells, and cells scraped from inside her mouth revealed not only 1 and 3 but also 2 and 4. It is not clear why her bone marrow was an exception - containing only **1,3** stem cells.
- How were these results possible? The most reasonable explanation is that
- Her mother had simultaneously ovulated **two eggs** one containing a chromosome 6 with HLA 3 and the other with HLA 4.
- Her father would, of course, have produced equal numbers of 1-containing and 2-containing sperm.
  - A 1-sperm fertilized the 3-egg.
  - A 2-sperm fertilized the 4-egg.
- Soon thereafter the resulting early embryos fused into a single embryo.
- As this embryo developed into a fetus, both types of cells participated in constructing her various organs including her oogonia (but not, apparently, the blood stem cells in her bone marrow).
- Although she was a mosaic for the HLA (and other) genes on chromosome 6, all her cells were XX. So both the father's successful sperm cells had carried his X chromosome.

However, tetraparental humans have been found that were mosaic for sex chromosomes as well; that is, some of their cells were XX; the other XY. In some cases this mosaic pattern results in a hermaphrodite - a person with a mixture of male and female sex organs.

So what are her chances for finding a suitable kidney donor?

The HLA region on chromosome 6 carries a set of genes that encode the major transplantation antigens; that is, the antigens that trigger graft rejection. Ordinarily, there is only a 1 in 4 chance that two siblings share the same transplantation antigens if both parents were heterozygous as in her case. But because this woman has all four sets of transplantation antigens, she can accept a kidney from any one of her brothers as well as her mother (her father was dead) without fear of rejecting it.Laboratory tests confirmed that she was unable to generate T cells able to react against the cells of either brother or her mother.

Rat-Mouse Chimeras

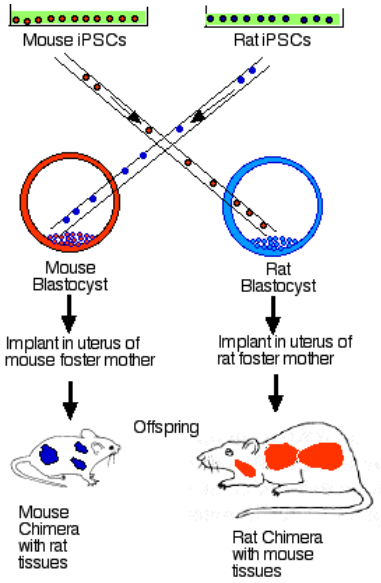


Figure 15.7.6.4 Rat-Mouse chimeras

In the 3 September 2010 issue of **Cell**, Kobayashi *et al.* report the creation of healthy rat-mouse chimeras:

- mice with functioning rat tissues
- rats with functioning mouse tissues.

Their procedure:

- Generate **induced pluripotent stem cells** (iPSCs) from embryonic fibroblasts of each species.
- Inject:
  - mouse iPSCs into rat blastocysts
  - rat iPSCs into mouse blastocysts.
- Implant these blastocysts into the uterus of pseudopregnant foster mothers of the same species as the blastocyst.

The *Pdx-1*$^{-/-}$ Mouse

Pdx-1 encodes a transcription factor that is essential for the development of the pancreas. Transgenic mice lacking a functioning *Pdx-1* gene (*Pdx-1*$^{-/-}$) die shortly after birth.

However, Kobayashi *et al.* found that injecting rat induced pluripotent stem cells (iPSCs) into mouse *Pdx-1*$^{-/-}$ blastocysts produced a few viable mouse chimeras complete with a pancreas made up almost exclusively of rat cells. The pancreas was fully

functional producing both exocrine secretions (e.g., pancreatic amylase) and endocrine secretions (e.g., insulin, glucagon, and somatostatin).

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15.7G: Human Cloning

This page discusses the successful cloning of Dolly the lamb by Dr. I. Wilmut's team in February 1997 at the Roslin Institute. The process involved fusing an adult sheep's cell nucleus with egg cells, leading to ethical debates surrounding cloning. Dolly's lineage was verified through DNA fingerprinting, ensuring no natural mating occurred with her surrogate. The exact reasons for the cloning success are still unclear, but may relate to the properties of the cultured cells used.

In February 1997, a research team at the Roslin Institute in Edinburgh, Scotland, headed by Dr. I. Wilmut, reported (in the 27 February 1997 issue of **Nature**) that they had succeeded in producing a healthy lamb, named **Dolly**, from the nucleus of a cell taken from an **adult** sheep.

Why has this achievement created such a stir?

After all, all the cells in an adult are

- descended from the fertilized egg
- have been produced by mitosis

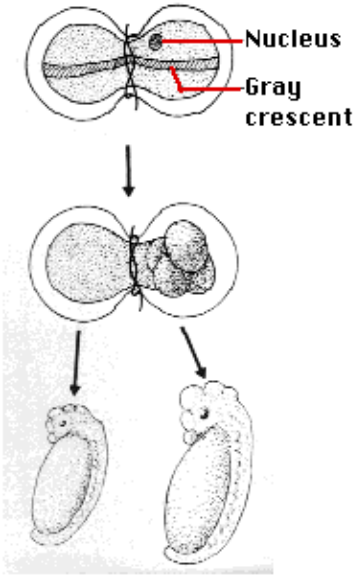


Figure 15.7.7.1 Gray crescent

Many years earlier, the German embryologist Hans Spemann showed that even after 5 divisions of the fertilized egg, the nuclei retained the potential to program the complete development of an adult. Using strands of baby hair, he tied loops around fertilized amphibian (newt) eggs so that they were constricted into two halves with the nucleus confined to one half and a narrow bridge of cytoplasm connecting the two halves.

He found that:

- At first only the half containing the zygote nucleus would divide by mitosis.
- Eventually a nucleus would cross into the other half and it, too, would begin dividing.
- So long as both halves contained some of a cytoplasmic region called the **gray crescent**, the second half would then go on to develop into a second perfectly-formed embryo.

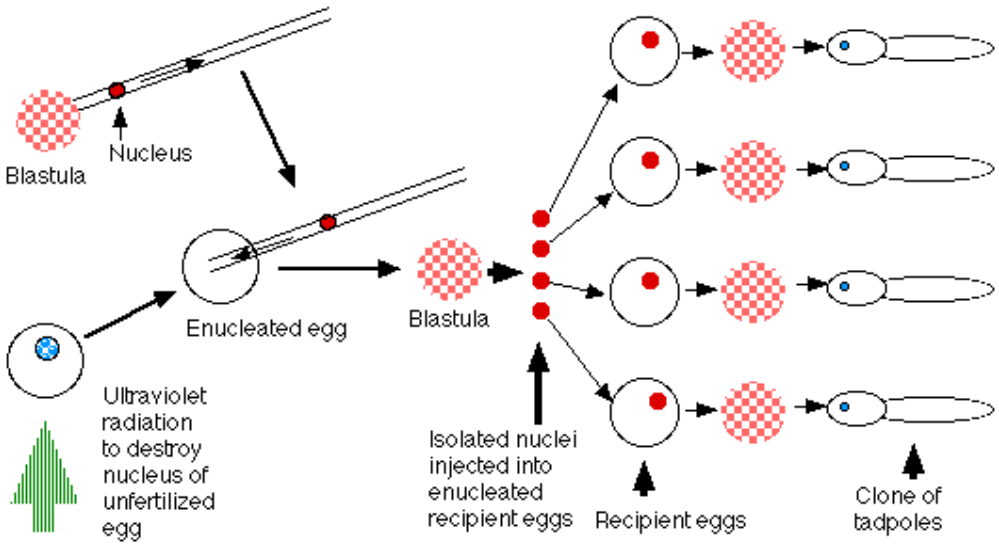


Figure 15.7.7.3 How Dolly was made

- Enucleate the eggs produced by Scottish Blackface ewes (female sheep).
  - Treat the ewes with gonadotropin-releasing hormone (GnRH) to cause them to produce oocytes ready to be fertilized. Like all mammals, these are arrested at metaphase of the second meiotic division (meiosis II).
  - Plunge a micropipette into the egg over the polar body and suck out not only the polar body but the haploid pronucleus within the egg.
- Fuse each enucleated egg with a diploid cell growing in culture.
  - Cells from the mammary gland of an adult Finn Dorset ewe (they have white faces) are grown in tissue culture.
  - Five days before use, the nutrient level in the culture is reduced so that the cells stop dividing and enter G_0 of the cell cycle.
  - Donor cells and enucleated recipient cells are placed together in culture.
  - The cultures are exposed to pulses of electricity to
    - cause their respective plasma membranes to fuse;
    - stimulate the resulting cell to begin mitosis (by mimicking the stimulus of fertilization).
- Culture the cells until they have grown into a morula (solid mass of cells) or even into a blastocyst (6 days).
- Transfer several of these into the uterus of each (of 13, in this case) Scottish Blackface ewes (previously treated with GnRH to prepare them for implantation.
- Wait (with your fingers crossed).

The result: One ewe gave birth (148 days later) to Dolly.

What made Dolly different?

The Wilmut group also used the same technique to produce healthy lambs using cells from lamb **embryos** (9 days after fertilization) and lamb **fetuses** (26 days after fertilization). But in these experiments, there was no way to know the phenotype of the nuclear donor because it had not yet been born. So, too, the recent cloning of monkeys from embryo nuclei represents simply an expansion of nature's ability to produce identical twins, etc. whose traits we will not know until they are born and grow up. But the nucleus that made Dolly came from an adult animal whose phenotypic traits were there to be seen.

How do we know that Dolly is not the progeny of an unsuspected mating of the foster mother?

- She has a white face and the foster mother is a Scottish Blackface
- DNA fingerprinting reveals bands found in Finn Dorset sheep (the breed that supplied the mammary cells), not those of Scottish Blackface sheep

What accounts for this remarkable achievement?

Besides years of hard work, we do not know. Perhaps:

- Using cells in G0 demethylates inactive genes and makes it possible once again for them to be expressed.
- The mammary gland cells were not truly differentiated epithelial cells but primitive stem cells present in the mammary gland.

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15.8: NERVOUS SYSTEM



- Topic hierarchy

15.8A: Neurons

15.8B: Synapses

15.8C: The Human Central Nervous System

15.8D: The Peripheral Nervous System

15.8E: Drugs and the Nervous System

15.8F: Nitric Oxide (NO)

15.8G: Prion Diseases

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15.8A: Neurons

Excitable cells are those that can be stimulated to create a tiny electric current. **Muscle fibers** and **nerve cells (neurons)** are excitable. The color photo is of a single inter**neuron** in the retina of a rabbit. The cell has been injected with a fluorescent dye to reveal all its branches. Each of the small knobs at the tips of the branches makes a **synapse** with another cell in the retina.

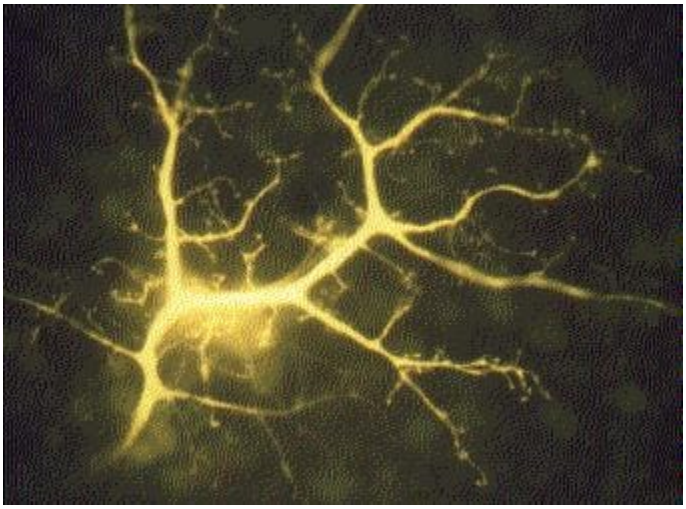


Figure 15.8.1.1: Neuron in a rabbit retina courtesy Julie H. Sandell and Richard H. Masland

The electric current in neurons is used to rapidly transmit signals through the animal, while the current in muscles is used to initiate contraction.

The Resting Potential

All cells (not just excitable cells) have a resting potential: an electrical charge across the plasma membrane, with the interior of the cell negative with respect to the exterior. The size of the resting potential varies, but in excitable cells runs about –70 millivolts (mv). The resting potential arises from two activities:

- The sodium/potassium ATPase: This pump pushes only two potassium ions (K^+) into the cell for every three sodium ions (Na^+) it pumps out of the cell so its activity results in a net loss of positive charges within the cell.
- Some potassium channels in the plasma membrane are "leaky" allowing a slow facilitated diffusion of K^+ out of the cell (red arrow).

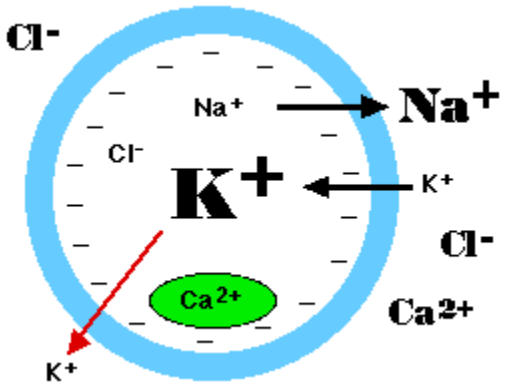


Figure 15.8.1.2: Resting potential

Ionic Relations in the Cell

The sodium/potassium ATPase produces

- a concentration of Na^+ outside the cell that is some 10 times greater than that inside the cell
- a concentration of K^+ inside the cell some 20 times greater than that outside the cell.

The concentrations of chloride ions (Cl^-) and calcium ions (Ca^{2+}) are also maintained at greater levels outside the cell EXCEPT that some intracellular membrane-enclosed compartments may also have high concentrations of Ca^{2+} (green oval).

Depolarization

Certain external stimuli reduce the charge across the plasma membrane.

- mechanical stimuli (e.g., stretching, sound waves) activate mechanically-gated sodium channels
- certain neurotransmitters (e.g., acetylcholine) open ligand-gated sodium channels

In each case, the facilitated diffusion of sodium into the cell reduces the resting potential at that spot on the cell creating an **excitatory postsynaptic potential** or **EPSP**. If the potential is reduced to the **threshold** voltage (about –50 mv in mammalian neurons), an action potential is generated in the cell.

Action Potentials

If depolarization at a spot on the cell reaches the threshold voltage, the reduced voltage now opens up hundreds of voltage-gated sodium channels in that portion of the plasma membrane. During the millisecond that the channels remain open, some 7000 Na^+ rush into the cell. The sudden complete depolarization of the membrane opens up more of the voltage-gated sodium channels in adjacent portions of the membrane. In this way, a wave of depolarization sweeps along the cell. This is the action potential. (In neurons, the action potential is also called the **nerve impulse**.)

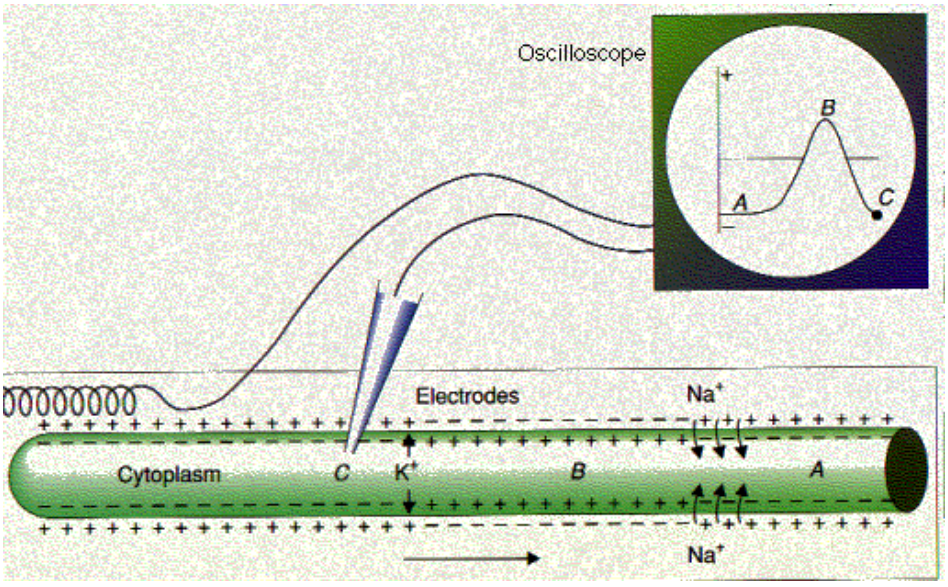


Figure 15.8.1.3 Action potential

The nerve impulse: (Figure 15.8.1.3) In the resting neuron, the interior of the axon membrane is negatively charged with respect to the exterior (**A**). As the action potential passes (**B**), the polarity is reversed. Then the outflow of K^+ ions quickly restores normal polarity (**C**). At the instant pictured in the diagram, the moving spot, which has traced these changes on the oscilloscope as the impulse swept past the intracellular electrode, is at position **C**.

The refractory period

A second stimulus applied to a neuron (or muscle fiber) less than 0.001 second after the first will not trigger another impulse. The membrane is depolarized (position **B**), and the neuron is in its **refractory period**. Not until the –70 mv polarity is reestablished (position **C**) will the neuron be ready to fire again. Repolarization is first established by the facilitated diffusion of **potassium ions** out of the cell. Only when the neuron is finally rested are the sodium ions that came in at each impulse actively transported back out of the cell. In some human neurons, the refractory period lasts only 0.001–0.002 second. This means that the neuron can transmit 500–1000 impulses per second.

The action potential is all-or-none

The strength of the action potential is an intrinsic property of the cell. So long as they can reach the threshold of the cell, strong stimuli produce no stronger action potentials than weak ones. However, the strength of the stimulus is encoded in the **frequency** of the action potentials that it generates.

Myelinated Neurons

The axons of many neurons are encased in a fatty sheath called the **myelin sheath**. It is the greatly expanded plasma membrane of an accessory cell called the Schwann cell. Where the sheath of one Schwann cell meets the next, the axon is unprotected. The voltage-gated sodium channels of myelinated neurons are confined to these spots (called **nodes of Ranvier**).

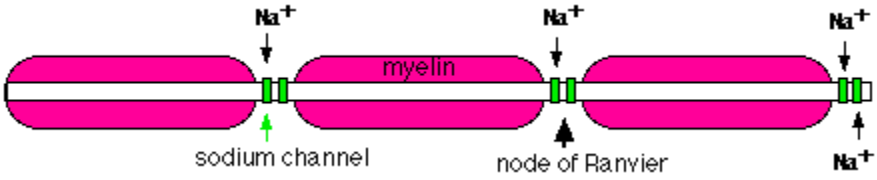


Figure 15.8.1.4 Nodes of Ranvier

The inrush of sodium ions at one node creates just enough depolarization to reach the threshold of the next. In this way, the action potential jumps from one node to the next. This results in much faster propagation of the nerve impulse than is possible in nonmyelinated neurons.

Multiple sclerosis

This autoimmune disorder results in the gradual destruction of myelin sheaths. Despite this, transmission of nerve impulses continues for a period as the cell inserts additional voltage-gated sodium channels in portions of the membrane formerly protected by myelin.

Hyperpolarization

Despite their name, some neurotransmitters **inhibit** the transmission of nerve impulses. They do this by opening **chloride channels** and/or **potassium channels** in the plasma membrane. In each case, opening of the channels **increases** the membrane potential by letting negatively-charged chloride ions (Cl^-) IN and positively-charged potassium ions (K^+) OUT. This hyperpolarization is called an **inhibitory postsynaptic potential** (**IPSP**) because it counteracts any excitatory signals that may arrive at that neuron. Although the **threshold** voltage of the cell is **unchanged**, it now requires a stronger excitatory stimulus to reach threshold.

Example: **Gamma amino butyric acid** (**GABA**). This neurotransmitter is found in the brain and inhibits nerve transmission by both mechanisms:

- binding to $GABA_A$ receptors opens chloride channels in the neuron
- binding to $GABA_B$ receptors opens potassium channels

Integrating Signals

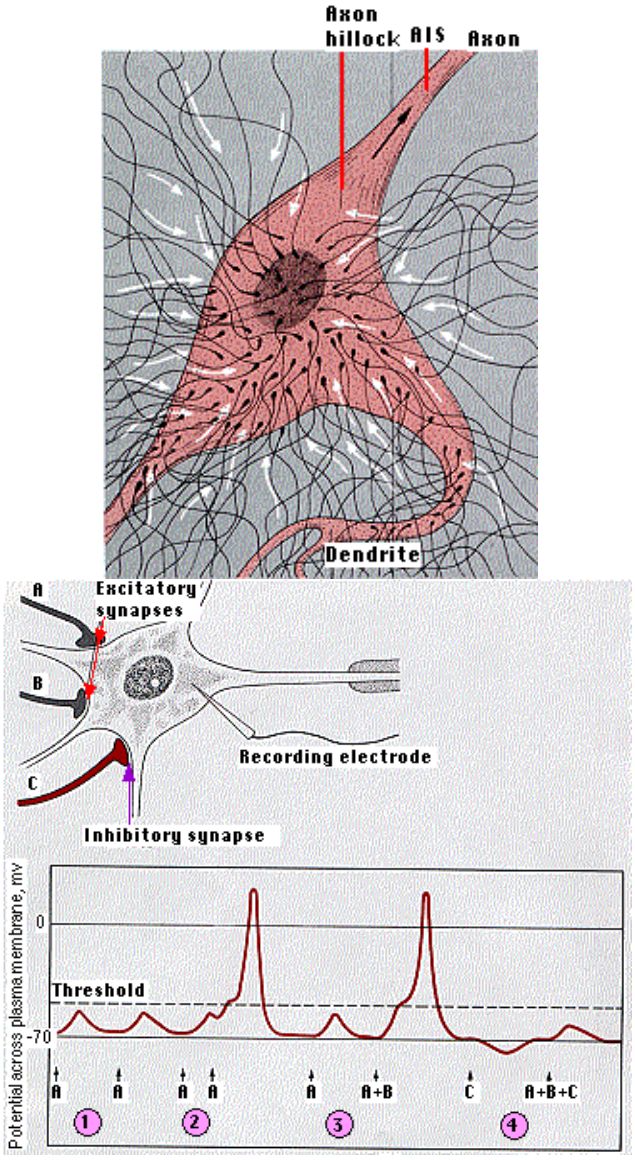


Figure 15.8.1.5 Neuron synapsing and EPSP

A single neuron, especially one in the central nervous system (see color photo at top), may have thousands of other neurons synapsing on it. Some of these release activating (depolarizing) neurotransmitters; others release inhibitory (hyperpolarizing) neurotransmitters.

The receiving cell is able to integrate these signals. The diagram shows how this works in a motor neuron.

- The EPSP created by a single excitatory synapse is insufficient to reach the threshold of the neuron.
- EPSPs created in quick succession, however, add together ("**summation**"). If they reach threshold, an action potential is generated.
- The EPSPs created by **separate** excitatory synapses (**A + B**) can also be added together to reach threshold.
- Activation of inhibitory synapses (**C**) makes the **resting potential** of the neuron more negative. The resulting **IPSP** may also prevent what would otherwise have been effective EPSPs from triggering an action potential.

Normally, the number of EPSPs needed to reach threshold is greater than shown here.

One might expect that depolarization at one point on the plasma membrane would generate an action potential irrespective of inhibitory signals elsewhere. However, this is avoided in many neurons by the **axon hillock** and the **axon initial segment** (the

AIS). This is the region where the axon emerges from the cell body and is unmyelinated. The portion of the plasma membrane in this region has few or no synapses of its own and a **lower threshold** than elsewhere on the cell.

Neurons can establish such distinctive domains on their plasma membrane by anchoring (with actin filaments) transmembrane proteins as barriers to block the free diffusion of membrane proteins from the cell body to the axon. The action potential is usually generated in the axon initial segment. Having neither excitatory nor inhibitory synapses of its own, it is able to evaluate the total picture of EPSPs and IPSPs created in the dendrites and cell body. Only if, over a brief interval, the sum of depolarizing signals minus the sum of the hyperpolarizing signals exceeds the threshold of the axon initial segment will an action potential be generated.

This method for the neuron to evaluate a mix of positive and negative signals occurs rapidly. It turns out, however, that neurons also have a long-term way to integrate a mix of positive and negative signals converging on them. This long-term response involves changes in gene activity leading to changes in the number and activity of the cell's many synapses.

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15.8B: Synapses

The coordination of cellular activities in animals is usually considered to involve the endocrine system (where the response is to hormones: chemicals secreted into the blood by endocrine glands and carried by the blood to the responding cell) and a nervous system (response to electrical impulses passing from the central nervous system to muscles and glands). However, in fact, coordination by the nervous system is also chemical. Most neurons achieve their effect by releasing chemicals, the neurotransmitters, on a receiving cell: another neuron (a "postsynaptic" neuron), a muscle cell and a gland cell. So the real distinction between nervous and endocrine coordination is that nervous coordination is faster and more localized. Neurotransmitters are chemicals that act in a paracrine fashion.

The junction between the axon terminals of a neuron and the receiving cell is called a synapse. Synapses at muscle fibers are also called neuromuscular junctions or myoneural junctions.

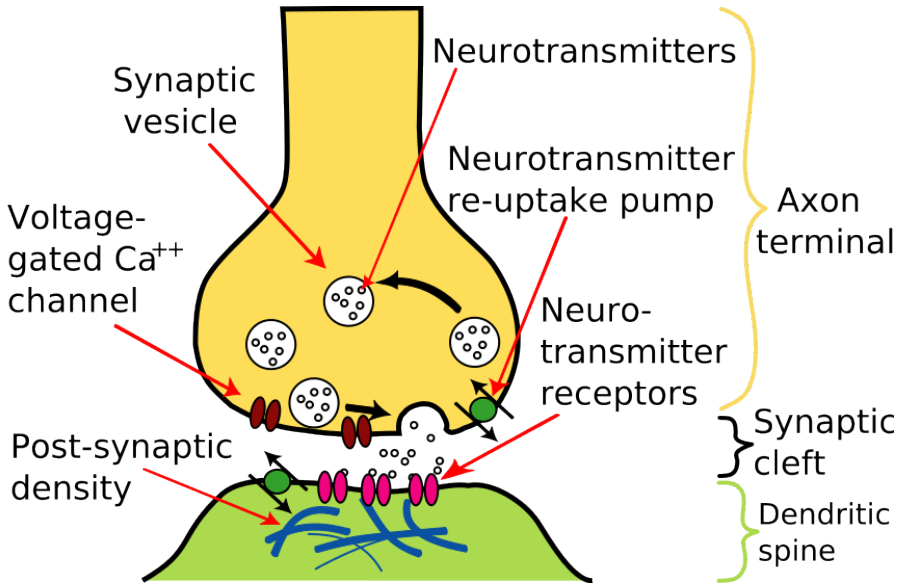


Figure 15.8.2.1: Synapses The neuron, synaptic transmission, and neurotransmitters. In R. M. Julien, A primer of drug action: A comprehensive guide to the actions, uses, and side effects of psychoactive drugs (pp. 60-88). New York, NY, USA: Worth Publishers. (CC-BY-SA-3.0; Nrets).

- Action potentials travel down the axon of the neuron to its end(s), the axon terminal(s).
- Each axon terminal is swollen forming a synaptic knob.
- The synaptic knob is filled with membrane-enclosed **vesicles** containing a neurotransmitter.
- Arrival of an action potential at the synaptic knob opens Ca^{2+} channels in the plasma membrane.
- The influx of Ca^{2+} triggers the **exocytosis** of some of the vesicles.
- Their neurotransmitter is released into the synaptic cleft.
- The neurotransmitter molecules bind to **receptors** on the postsynaptic membrane.
- These receptors are ligand-gated **ion channels**.

Excitatory synapses

The neurotransmitter at excitatory synapses **depolarizes** the postsynaptic membrane (of a neuron in this diagram). Example: **acetylcholine** (**ACh**)

- Binding of acetylcholine to its receptors on the postsynaptic cell opens up ligand-gated **sodium channels**.
- These allow an influx of Na^{+} ions, reducing the membrane potential.
- This reduced membrane potential is called an **excitatory postsynaptic potential** or **EPSP**.
- If depolarization of the postsynaptic membrane reaches threshold, an **action potential** is generated in the postsynaptic cell.

Inhibitory synapses

The neurotransmitter at inhibitory synapses **hyperpolarizes** the postsynaptic membrane. Example: **gamma aminobutyric acid** (**GABA**) at certain synapses in the brain.

The $GABA_A$ receptor is a ligand-gated chloride channel. Binding of GABA to the receptors increases the influx of chloride (Cl^-) ions into the postsynaptic cell raising its membrane potential and thus inhibiting it. (The neurotransmitter glycine acts in the same way at synapses in the spinal cord and the base of the brain.) This is a **fast** response - taking only about 1 millisecond. Binding of GABA to $GABA_B$ receptors activates an internal **G protein** and a "second messenger" that leads to the opening of nearby potassium (K^+) channels. As you might expect, this is a slower response, taking as long as 1 second.

In both cases, the resulting facilitated diffusion of ions (chloride IN; potassium OUT) **increases** the membrane potential (to as much as −80 mv). This increased membrane potential is called an **inhibitory postsynaptic potential** (**IPSP**) because it counteracts any excitatory signals that may arrive at that neuron. A hyperpolarized neuron appears to have an increased threshold. Actually, the threshold voltage (about −50 mv) has not changed. It is simply a question of whether the depolarization produced by excitatory synapses on the cell **minus** the hyperpolarizing effect of inhibitory synapses can reach this value or not.

Neurotransmitters

Acetylcholine (ACh)

Widely used at synapses in the peripheral nervous system. Released at the terminals of

- all motor neurons activating **skeletal muscle**.
- all preganglionic neurons of the **autonomic nervous system.**
- the postganglionic neurons of the **parasympathetic** branch of the autonomic nervous system.

Also mediates transmission at some synapses in the brain. These include synapses involved in the acquisition of short-term memory. Drugs that enhance ACh levels - acetylcholinesterase inhibitors - are now used in elderly patients with failing memory (e.g., Alzheimer's patients).

Nicotinic vs. Muscarinic Acetylcholine Receptors

ACh acts on two different types of receptor:

- Nicotinic receptors are
  - found at the neuromuscular junction of skeletal (only) muscles
  - on the post-ganglionic neurons of the parasympathetic nervous system
  - on many neurons in the brain (e.g. neurons in the hypothalamus whose activation by nicotine suppresses appetite)
  - Nicotine is an agonist (hence the name)
  - Curare is an antagonist (hence its ability to paralyze skeletal muscles)
- Muscarinic receptors are
  - Found at the neuromuscular junctions of cardiac and smooth muscle as well as on glands and on the post-ganglionic neurons of the sympathetic nervous system.
  - Muscarine (a toxin produced by certain mushrooms) is an agonist.
  - Atropine is an antagonist (hence its use in acetylcholinesterase poisoning).

Amino acids

- **Glutamic acid** (Glu); used at **excitatory synapses** in the central nervous system (**CNS**). Essential for **long term potentiation** (**LTP**), a form of memory.
  Like GABA, Glu acts on two types of CNS synapses:
  - FAST (~1 msec) with Glu opening ligand-gated Na^+ channels;
  - SLOW (~1 sec) with Glu binding to G-protein-coupled receptors receptors that turn on a "second messenger" cascade of

biochemical changes that open channels allowing Na^+ into the cell.
- **Gamma aminobutyric acid** (**GABA**); used at inhibitory synapses in the **CNS**.
- **Glycine** (**Gly**). Also used at inhibitory synapses in the CNS. In fact, both GABA and glycine are released together at some inhibitory synapses.

Catecholamines

Synthesized from tyrosine (Tyr).

- **Noradrenaline** (also called norepinephrine). Released by **postganglionic neurons** of the sympathetic branch of the **autonomic nervous system**. Also used at certain synapses in the CNS.
- **Dopamine**. Used at certain synapses in the CNS.

Other monoamines

- **Serotonin** (also known as 5-hydroxytryptamine or 5HT). Synthesized from tryptophan (Trp).
- **Histamine**

Both of these neurotransmitters are confined to synapses in the brain. However, serotonin is also secreted from the duodenum, where it acts in a paracrine manner to stimulate intestinal peristalsis, and as a circulating hormone, where it is taken up by platelets and also suppresses bone formation.

Peptides

A selection of 9 of the 40 or more peptides that are suspected to serve as neurotransmitters in the brain. The first six also serve as **hormones**.

- Vasopressin
- Oxytocin
- Somatostatin
- Gonadotropin-releasing hormone (GnRH)
- Angiotensin II
- Cholecystokinin (CCK)
- Substance P
- Two **enkephalins**
  - Met-enkephalin (Tyr-Gly-Gly-Phe-Met)
  - Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu)

ATP

ATP - probably along with another neurotransmitter is released at some synapses in the brain.

Synaptic Plasticity

Most neurons release a single neurotransmitter at the synapses at their axon terminals. However, some exceptions have been found.

- neurons that release one transmitter at some of their terminals, a different one at others
- neurons that switch from one neurotransmitter to a different one when the stimulus reaching them changes. Example: interneurons in the hypothalamus of the rat that release dopamine when the rats are exposed to short-day photoperiods (which these nocturnal animals like) but switch to releasing somatostatin when the rats are exposed to long days (which they don't like).

Turning Synapses Off

Once its job is done, the neurotransmitter must be removed from the synaptic cleft to prepare the synapse for the arrival of the next action potential. Two methods are used:

- **Reuptake**. The neurotransmitter is taken back into the synaptic knob of the presynaptic neuron by active transport. All the neurotransmitters **except** acetylcholine use this method.
- **Acetylcholine** is removed from the synapse by enzymatic breakdown into inactive fragments. The enzyme used is **acetylcholinesterase**.

  Nerve gases used in warfare (e.g., sarin) and the organophosphate insecticides (e.g., parathion) achieve their effects by inhibiting acetylcholinesterase thus allowing ACh to remain active. Atropine is used as an antidote because it blocks ACh muscarinic receptors.

Drugs and Synapses

Many drugs that alter mental state achieve at least some of their effects by acting at synapses.

GABA Receptors

The $GABA_A$ receptor is a ligand-gated chloride channel. Activation of the receptors increases the influx of chloride (Cl^-) ions into the postsynaptic cell raising its membrane potential and thus inhibiting it. A number of drugs bind to the $GABA_A$ receptor. They bind at sites different from the spot where GABA itself binds, but increase the strength of GABA's binding to its site. Thus they enhance the inhibitory effect of GABA in the CNS. These drugs include **sedatives** like phenobarbital and anti-anxiety drugs like Valium, Librium, Halcion (all members of a group called benzodiazepines). In view of their common action, it is not surprising that they act additively; taken together these drugs can produce dangerous overdoses. The recreational (and illegal) drug γ-hydroxybutyrate binds to the $GABA_B$ receptor.

Catecholamine synapses

Many antidepressant drugs (the so-called tricyclic antidepressants like amitriptyline ["Elavil"]) interfere with the **reuptake** of **noradrenaline** and **serotonin** from their synapses and thus enhance their action at the synapse. The popular antidepressant fluoxetine ("**Prozac**"), seems to block only the reuptake of serotonin.

Dopamine synapses

One class of dopamine receptor is bound by such drugs as chlorpromazine and haloperidol. Binding of these drugs leads to increased synthesis of dopamine at the synapse and eases some of the symptoms of **schizophrenia**.

Synapses blocking pain signals

The two **enkephalins** are released at synapses on neurons involved in transmitting pain signals back to the brain. The enkephalins **hyperpolarize** the postsynaptic membrane thus inhibiting it from transmitting these pain signals. The ability to perceive pain is vital. However, faced with massive, chronic, intractable pain, it makes sense to have a system that decreases its own sensitivity. Enkephalin synapses provide this intrinsic pain-suppressing system.

Opiates such as heroin, morphine, codeine, and methadone bind these same receptors. This makes them excellent pain killers. However, they are also highly **addictive**.

- By binding to enkephalin receptors, they enhance the pain-killing effects of the enkephalins.
- A homeostatic reduction in the sensitivity of these synapses compensates for continued exposure to opiates.
- This produces **tolerance**, the need for higher doses to achieve the prior effect.
- If use of the drug ceases, the now relatively insensitive synapses respond less well to the soothing effects of the enkephalins, and the painful symptoms of **withdrawal** are produced.

Electrical Synapses

Electrical synapses are a rare exception to the general rule that neurons signal other neurons by release of chemical neurotransmitters. Some properties of electrical synapses:

- The two neurons are connected by gap junctions, the space between them being much smaller (~2 nm) than that at chemical synapses (~20 nm).
- In many cases, transmission of action potentials can pass in either direction.
- Transmission between neurons is as much as ten times faster than in chemical synapses.
- Thus electrical synapses can mediate, for example, the very rapid escape response of crayfish that encounter a threat.
- Electrical synapses in the CNS permit groups of interneurons to fire together.
- They are found in vertebrates (e.g. in the hippocampus of the brain) as well as in invertebrates like the crayfish.

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15.8C: The Human Central Nervous System

The central nervous system is made up of the spinal cord and brain. The spinal cord conducts sensory information from the peripheral nervous system (both somatic and autonomic) to the brain. It also conducts motor information from the brain to our various effectors: skeletal muscles, cardiac muscle, smooth muscle, glands, and serves as a minor reflex center. The brain receives sensory input from the spinal cord as well as from its own nerves (e.g., olfactory and optic nerves) and devotes most of its volume (and computational power) to processing its various sensory inputs and initiating appropriate and coordinated motor outputs

White Matter vs. Gray Matter

Both the spinal cord and the brain consist of *white matter* (bundles of **axons** each coated with a sheath of myelin) and gray matter (masses of the **cell bodies** and **dendrites** each covered with synapses). In the spinal cord, the white matter is at the surface, the gray matter inside. In the brain of **mammals**, this pattern is reversed. However, the brains of "lower" vertebrates like fishes and amphibians have their white matter on the outside of their brain as well as their spinal cord.

The Meninges

Both the spinal cord and brain are covered in three continuous sheets of connective tissue, the meninges. From outside in, these are thedura mater — pressed against the bony surface of the interior of the vertebrae and the cranium, the arachnoid, nd the pia mater. The region between the arachnoid and pia mater is filled with cerebrospinal fluid (CSF).

The Interstitial Fluid of the Central Nervous System

The cells of the central nervous system are bathed in a fluid, called **cerebrospinal fluid** (**CSF**), that differs from that serving as the interstitial fluid (ISF) of the cells in the rest of the body. Cerebrospinal fluid leaves the capillaries in the choroid plexus of the brain. It contains far less protein than "normal" because of the **blood-brain barrier**, a system of tight junctions between the endothelial cells of the capillaries. This barrier creates problems in medicine as it prevents many therapeutic drugs from reaching the brain. CSF flows uninterrupted throughout the central nervous system through the central **cerebrospinal canal** of the spinal cord and through an interconnected system of four **ventricles** in the brain.

CSF returns to the blood through lymphatic vessels draining the brain.In mice, the flow of CSF increases by 60% when they are asleep. Perhaps one function of sleep is to provide the brain a way of removing potentially toxic metabolites accumulated during waking hours.

The Spinal Cord

31 pairs of **spinal nerves** arise along the spinal cord. These are **"mixed" nerves** because each contain both sensory and motor axons. However, within the spinal column, all the **sensory axons** pass into the **dorsal root ganglion** where their cell bodies are located and then on into the spinal cord itself. All the **motor axons** pass into the **ventral roots** before uniting with the sensory axons to form the mixed nerves.

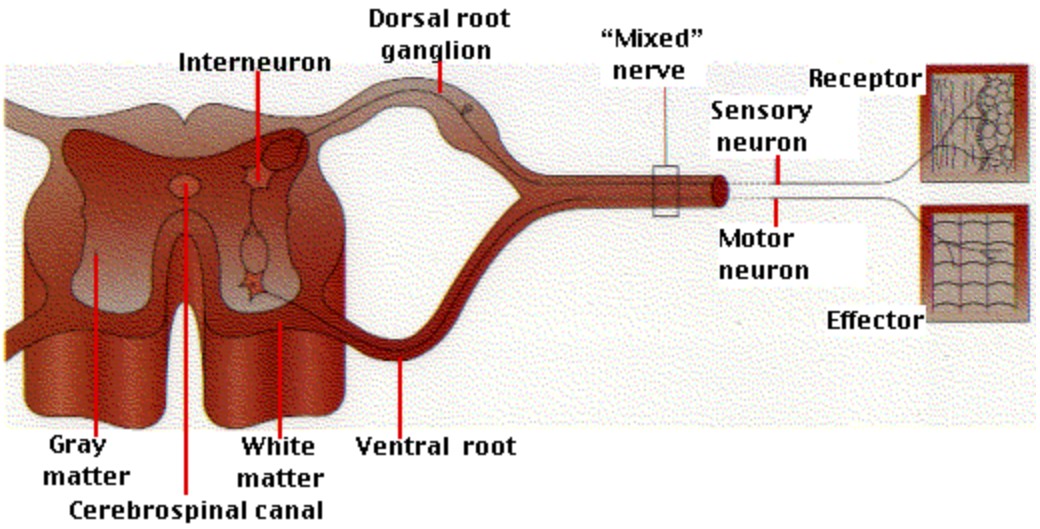


Figure 15.8.3.1 Spinal cord

The spinal cord carries out two main functions:

- It connects a large part of the peripheral nervous system to the brain. Information (nerve impulses) reaching the spinal cord through sensory neurons are transmitted up into the brain. Signals arising in the motor areas of the brain travel back down the cord and leave in the motor neurons.
- The spinal cord also acts as a minor coordinating center responsible for some simple reflexes like the withdrawal reflex.

The interneurons carrying impulses to and from specific receptors and effectors are grouped together in **spinal tracts**.

Crossing Over of the Spinal Tracts

Impulses reaching the spinal cord from the left side of the body eventually pass over to tracts running up to the right side of the brain and vice versa. In some cases this crossing over occurs as soon as the impulses enter the cord. In other cases, it does not take place until the tracts enter the brain itself.

The Brain

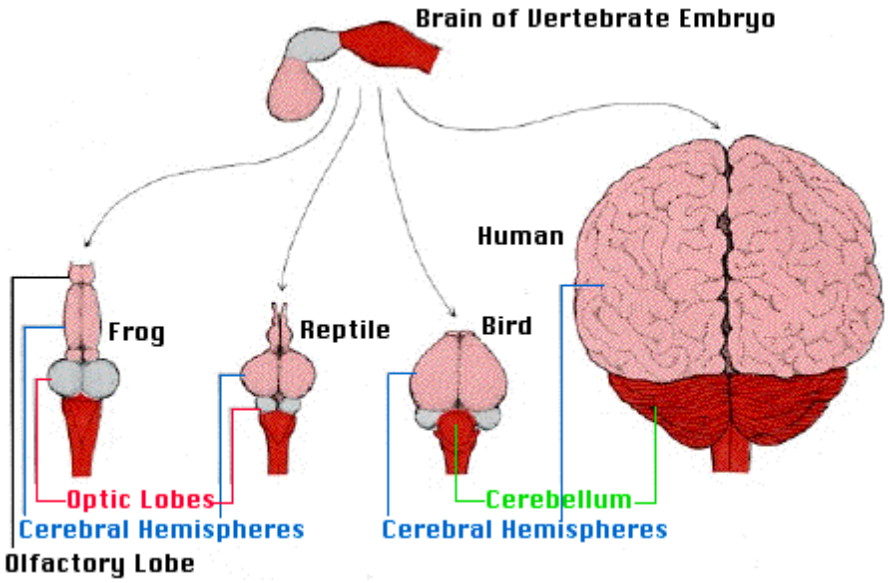


Figure 15.8.3.2 Brain of vertebrates

The brain of all vertebrates develops from three swellings at the anterior end of the neural tube of the embryo. From front to back

these develop into the

- **forebrain** (also known as the prosencephalon — shown in light color)
- **midbrain** (mesencephalon — gray)
- **hindbrain** (rhombencephalon — dark color) The human brain is shown from behind so that the cerebellum can be seen.

The human brain receives nerve impulses from the spinal cord and 12 pairs of **cranial nerves:**

- Some of the cranial nerves are "mixed", containing both sensory and motor axons
- Some, e.g., the optic and olfactory nerves (numbers I and II) contain sensory axons only
- Some, e.g. number III that controls eyeball muscles, contain motor axons only.

The Hindbrain

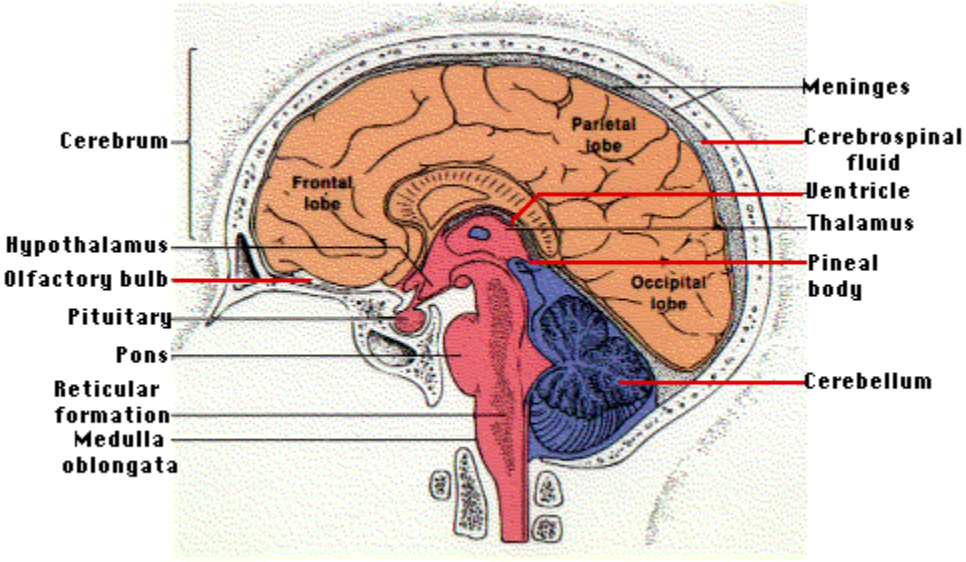


Figure 15.8.3.3 The Hindbrain

The main structures of the hindbrain (rhombencephalon) are the medulla oblongata, pons and cerebellum.

Medulla oblongata

The medulla looks like a swollen tip to the spinal cord. Nerve impulses arising here rhythmically stimulate the intercostal muscles and diaphragm thus making breathing possible. It also regulate heartbeats and regulate the diameter of arterioles thus adjusting blood flow. The neurons controlling breathing have **mu (μ) receptors**, the receptors to which **opiates**, like heroin, bind. This accounts for the suppressive effect of opiates on breathing. Destruction of the medulla causes instant death.

Pons

The pons seems to serve as a relay station carrying signals from various parts of the cerebral cortex to the cerebellum. Nerve impulses coming from the eyes, ears and touch receptors are sent on the cerebellum. The pons also participates in the reflexes that regulate breathing.

The **reticular formation** is a region running through the middle of the hindbrain (and on into the midbrain). It receives sensory input (e.g., sound) from higher in the brain and passes these back up to the thalamus. The reticular formation is involved in sleep, arousal (and vomiting).

Cerebellum

The cerebellum consists of two deeply-convoluted hemispheres. Although it represents only 10% of the weight of the brain,

it contains as many neurons as all the rest of the brain combined. Its most clearly-understood function is to coordinate body movements. People with damage to their cerebellum are able to perceive the world as before and to contract their muscles, but their motions are jerky and uncoordinated. So the cerebellum appears to be a center for learning motor skills (implicit memory). Laboratory studies have demonstrated both long-term potentiation (**LTP**) and long-term depression (**LTD**) in the cerebellum.

The Midbrain

The midbrain (mesencephalon) occupies only a small region in humans (it is relatively much larger in "lower" vertebrates). We shall look at only three features:

- the **reticular formation**: collects input from higher brain centers and passes it on to motor neurons.
- the **substantia nigra**: helps "smooth" out body movements; damage to the substantia nigra causes Parkinson's disease.
- the **ventral tegmental area** (**VTA**): packed with **dopamine**-releasing neurons that are activated by nicotinic acetylcholine receptors and whose projections synapse deep within the forebrain.The VTA seems to be involved in pleasure: nicotine, amphetamines and cocaine bind to and activate its dopamine-releasing neurons and this may account at least in part for their addictive qualities.

The midbrain along with the medulla and pons are often referred to as the "brainstem".

The Forebrain

The human forebrain (prosencephalon) is made up of a pair of large **cerebral hemispheres**, called the **telencephalon**. Because of crossing over of the spinal tracts, the left hemisphere of the forebrain deals with the right side of the body and vice versa. A group of structures located deep within the cerebrum make up the **diencephalon**.

Diencephalon

We shall consider four of its structures:

- **Thalamus**.
  - All **sensory** input (except for olfaction) passes through these paired structures on the way up to the somatic-sensory regions of the cerebral cortex and then returns to them from there.
  - Signals from the cerebellum pass through them on the way to the motor areas of the cerebral cortex.
- **Lateral geniculate nucleus** (**LGN**). All signals entering the brain from each optic nerve enter a LGN and undergo some processing before moving on the various visual areas of the cerebral cortex.
- **Hypothalamus**.
  - The seat of the autonomic nervous system. Damage to the hypothalamus is quickly fatal as the normal homeostasis of body temperature, blood chemistry, etc. goes out of control.
  - The source of 8 hormones, two of which pass into the posterior lobe of the pituitary gland.
- **Posterior lobe of the pituitary**.
  Receives vasopressin and oxytocin from the hypothalamus and releases them into the blood.

The Cerebral Hemispheres

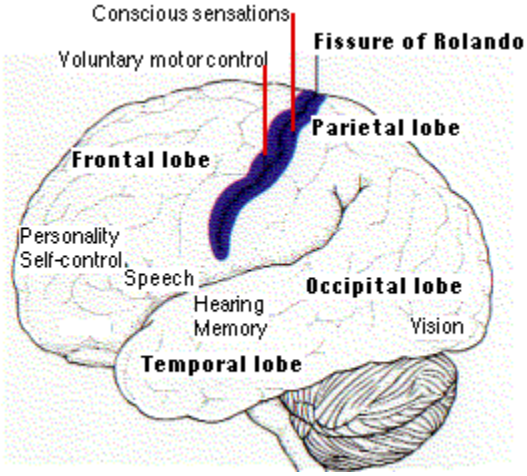


Figure 15.8.3.4 The Cerebral hemisphere

Each hemisphere of the cerebrum is subdivided into four lobes visible from the outside:

- **frontal**
- **parietal**
- **occipital**
- **temporal**

Hidden beneath these regions of each cerebral cortex is

- An **olfactory bulb**; they receive input from the olfactory epithelia.
- A **striatum**; they receive input from the frontal lobes and also from the limbic system (below). At the base of each striatum is a **nucleus accumbens** (NA).

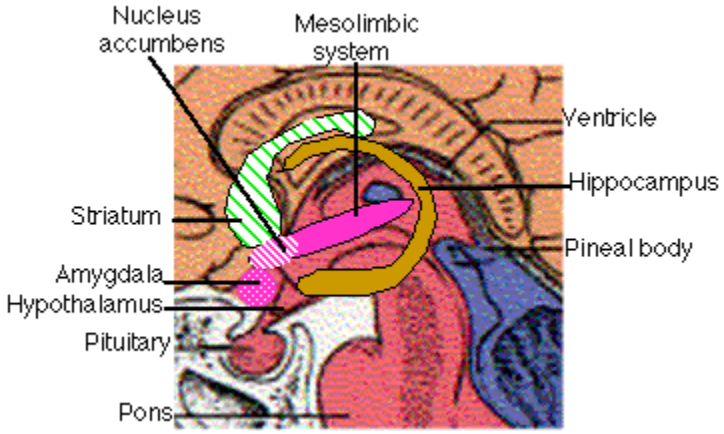


Figure 15.8.3.5 Striatum

The pleasurable (and addictive) effects of amphetamines, cocaine, and perhaps other psychoactive drugs seem to depend on their producing increasing levels of dopamine at the synapses in the nucleus accumbens (as well as the VTA).

- a **limbic system**; they receives input from various association areas in the cerebral cortex and pass signals on to the **nucleus accumbens**. Each limbic system is made up of a:
  - **hippocampus**. It is essential for the formation of long-term memories.
  - The amygdala appears to be a center of emotions (e.g., fear). It sends signals to the hypothalamus and medulla which can activate the flight or fight response of the autonomic nervous system. In rats, at least, the amygdala contains receptors for
    - vasopressin whose activation increases aggressiveness and other signs of the flight or fight response
    - oxytocin whose activation lessens the signs of stress

The amygdala receives a rich supply of signals from the olfactory system, and this may account for the powerful effect that odor has on emotions (and evoking memories).

Mapping the Functions of the Brain

It is estimated that the human brain contains some 86 billion (8.6×10^{10}) neurons averaging 10,000 synapses on each; that is, almost 10^{15} connections. How to unravel the workings of such a complex system?

Several methods have been useful.

Histology

Microscopic examination with the aid of selective stains has revealed many of the physical connections created by axons in the brain.

The Electroencephalograph (EEG)

This device measures electrical activity (brain "waves") that can be detected at the surface of the scalp. It can distinguish between, for example, sleep and excitement. It is also useful in diagnosing brain disorders such as a tendency to epileptic seizures.

Damage to the Brain

Many cases of brain damage from, for example,

- strokes (interruption of blood flow to a part of the brain)
- tumors in the brain
- mechanical damage (e.g., bullet wounds)

have provided important insights into the functions of various parts of the brain.

Example 1:

Battlefield injury to the left temporal lobe of the cerebrum interferes with speech.

Example 2: Phineas P. Gage

In 1848, an accidental explosion drove a metal bar completely through the frontal lobes of Phineas P. Gage. Not only did he survive the accident, he never even lost consciousness or any of the clearly-defined functions of the brain. However, over the ensuing years, he underwent a marked change in personality. Formerly described as a reasonable, sober, conscientious person, he became — in the words of those observing him — "thoughtless, irresponsible, fitful, obstinate, and profane". In short, his personality had changed, but his vision, hearing, other sensations, speech, and body coordination were unimpaired. (Similar personality changes have since been often observed in people with injuries to their prefrontal cortex.)



Figure 15.8.3.6 Gage skull

The photograph (courtesy of the Warren Anatomical Museum, Harvard University Medical School) shows Gage's skull where the bar entered (left) and exited (right) in the accident (which occurred 12 years before he died of natural causes in 1861).

Stimulating the exposed brain with electrodes

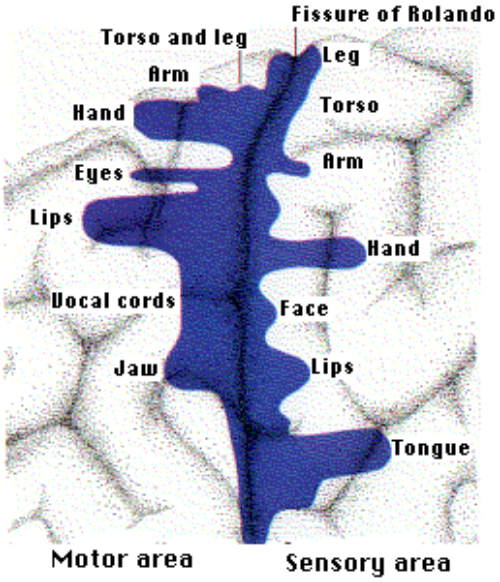


Figure 15.8.3.7 Motor and sensory area

There are no pain receptors on the surface of the brain, and some humans undergoing brain surgery have volunteered to have their exposed brain stimulated with electrodes during surgery. When not under general anesthesia, they can even report their sensations to the experimenter. Experiments of this sort have revealed a band of cortex running parallel to and just in front of the **fissure of Rolando** that controls the contraction of skeletal muscles. Stimulation of tiny spots within this **motor area** causes contraction of the muscles.

The area of motor cortex controlling a body part is not proportional to the size of that part but is proportional to the number of motor neurons running to it. The more motor neurons that activate a structure, the more precisely it can be controlled. Thus the areas of the motor cortex controlling the hands and lips are much larger than those controlling the muscles of the torso and legs. A similar region is located in a parallel band of cortex just behind the fissure of Rolando. This region is concerned with **sensation** from the various parts of the body. When spots in this **sensory area** are stimulated, the patient reports sensations in a specific area of the body. A map can be made based on these reports. When portions of the **occipital lobe** are stimulated electrically, the patient reports light. However, this region is also needed for associations to be made with what is seen. Damage to regions in the occipital lobe results in the person's being perfectly able to see objects but incapable of recognizing them.

The centers of hearing — and understanding what is heard — are located in the **temporal lobes**.

CT = X-ray C omputed T omography

This is an imaging technique that uses a series of X-ray exposures taken from different angles. Computer software can integrate these to produce a three-dimensional picture of the brain (or other body region). CT scanning is routinely used to quickly diagnose strokes.

PET = P ositron-E mission T omography

This imaging technique requires that the subject be injected with a radioisotope that emits positrons.

- Water labeled with **oxygen-15** ($H_2{}^{15}O$) is used to measure changes in blood flow (which increases in parts of the brain that are active). The short half-life of ^{15}O (2 minutes) makes it safe to use.

- **Deoxyglucose** labeled with fluorine-18. The brain has a voracious appetite for glucose (although representing only ~2% of our body weight, the brain receives ~15% of the blood pumped by the heart and consumes ~20% of the energy produced by cellular respiration when we are at rest). When supplied with deoxyglucose, the cells are tricked into taking in this related molecule and phosphorylating it in the first step of glycolysis. But no further processing occurs so it accumulates in the cell. By coupling a short-lived radioactive isotope like ^{18}F to the deoxyglucose and using a PET scanner, it is possible to visualize active regions of the brain.

The images in fig. 15.8.3.8 (courtesy of Michael E. Phelps from **Science 211**:445, 1981) were produced in a PET scanner. The **dark** areas are regions of high metabolic activity. Note how the metabolism of the occipital lobes (arrows) increases when visual stimuli are received.

Similarly, sounds increase the rate of deoxyglucose uptake in the speech areas of the temporal lobe.

The image in fig. 15.8.3.9 on the right (courtesy of Gary H. Duncan from Talbot, J. D., et. al., **Science 251**: 1355, 1991) shows activation of the cerebral cortex by a hot probe (which the subjects describe as painful) applied to the forearm.

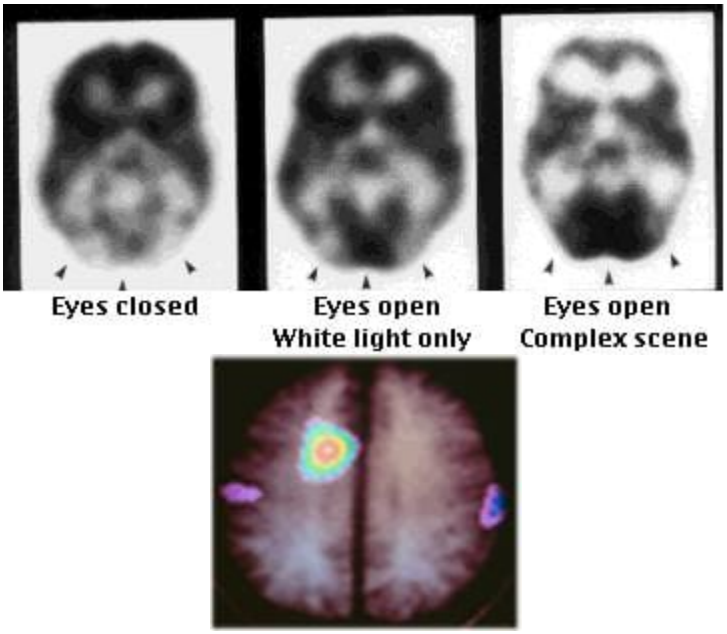


Figure 15.8.3.9 Cerebral cortex activation

Most cancers consume large amounts of glucose (cellular respiration is less efficient than in normal cells so they must rely more on the inefficient process of glycolysis). Therefore PET scanning with ^{18}F-fluorodeoxyglucose is commonly used to monitor both the primary tumor and any metastases.

MRI = M agnetic R esonance I maging

This imaging technique uses powerful magnets to detect magnetic molecules within the body. These can be endogenous molecules or magnetic substances injected into a vein.

fMRI = Functional Magnetic Resonance Imaging

fMRI exploits the changes in the magnetic properties of hemoglobin as it carries oxygen. Activation of a part of the brain increases oxygen levels there increasing the ratio of oxyhemoglobin to deoxyhemoglobin.

The probable mechanism:

- The increased demand for neurotransmitters must be met by increased production of ATP.
- Although this consumes oxygen (needed for cellular respiration),

- it also increases the blood flow to the area.
- So there is an increase and not a decrease in the oxygen supply to the region, which provides the signal detected by fMRI.

Magnetoencephalography (MEG)

MEG detects the tiny magnetic fields created as individual neurons "fire" within the brain. It can pinpoint the active region with a millimeter, and can follow the movement of brain activity as it travels from region to region within the brain. MEG is noninvasive requiring only that the subject's head lie within a helmet containing the magnetic sensors.

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15.8D: The Peripheral Nervous System

This page explains the division of the nervous system into the central (CNS) and peripheral nervous systems (PNS). The PNS is further categorized into the sensory-somatic and autonomic systems, which include cranial and spinal nerves for sensory and motor functions. The autonomic nervous system splits into sympathetic (emergency response) and parasympathetic (restoration) systems, relying on neurotransmitters like acetylcholine and noradrenaline.

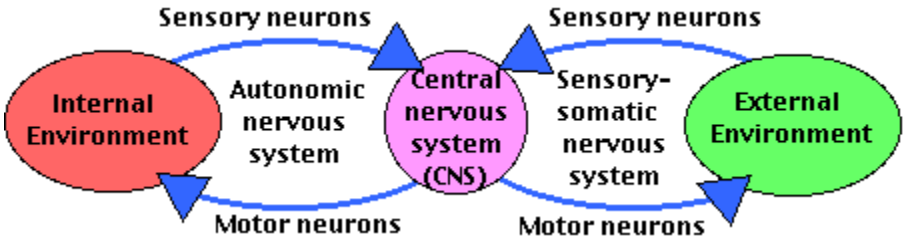


Figure 15.8.4.1 Nervous system

The nervous system is divided into the **peripheral nervous system** (**PNS**) and the **central nervous system** (**CNS**).
The **PNS** consists of

- **sensory neurons** running from stimulus **receptors** that inform the CNS of the stimuli
- **motor neurons** running from the CNS to the **muscles and glands** - called **effectors** - that take action.

The **CNS** consists of the **spinal cord** and the **brain.**

The **peripheral nervous system** is subdivided into the

- **sensory-somatic nervous system** and the
- **autonomic nervous system**

The Sensory-Somatic Nervous System

The sensory-somatic system consists of 12 pairs of **cranial nerves** and 31 pairs of **spinal nerves**.

The Cranial Nerves

Nerves	**Type**	**Function**
I Olfactory	sensory	olfaction (smell)
II Optic	sensory	vision (Contain 38% of all the axons connecting to the brain.)
III Oculomotor	motor*	eyelid and eyeball muscles
IV Trochlear	motor*	eyeball muscles
V Trigeminal	mixed	Sensory: facial and mouth sensation Motor: chewing
VI Abducens	motor*	eyeball movement
VII Facial	mixed	Sensory: taste Motor: facial muscles and salivary glands

VIII Auditory	sensory	hearing and balance
IX Glossopharyngeal	mixed	Sensory: taste Motor: swallowing
X Vagus	mixed	main nerve of the parasympathetic nervous system (PNS)
XI Accessory	motor	swallowing; moving head and shoulder
XII Hypoglossal	motor*	tongue muscles

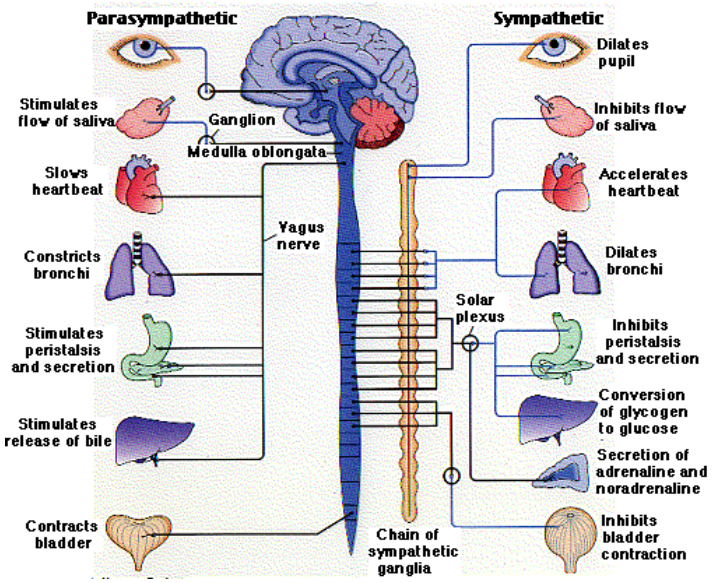


Figure 15.8.4.2 Autonomic nervous system

The autonomic nervous system consists of sensory neurons and motor neurons that run between the central nervous system (especially the **hypothalamus** and **medulla oblongata**) and various internal organs such as the heart, lungs, viscera and the glands (both exocrine and endocrine). It is responsible for monitoring conditions in the internal environment and bringing about appropriate changes in them. The contraction of both smooth muscle and cardiac muscle is controlled by motor neurons of the autonomic system. The actions of the autonomic nervous system are largely **involuntary** (in contrast to those of the sensory-somatic system). It also differs from the sensory-somatic system as it is using two groups of motor neurons to stimulate the effectors instead of one. First, the **preganglionic neurons** arise in the CNS and run to a ganglion in the body. Here they synapse with **postganglionic neurons**, which run to the effector organ (cardiac muscle, smooth muscle, or a gland).

The autonomic nervous system has two subdivisions:

- **sympathetic nervous system**
- **parasympathetic nervous system**.

The Sympathetic Nervous System

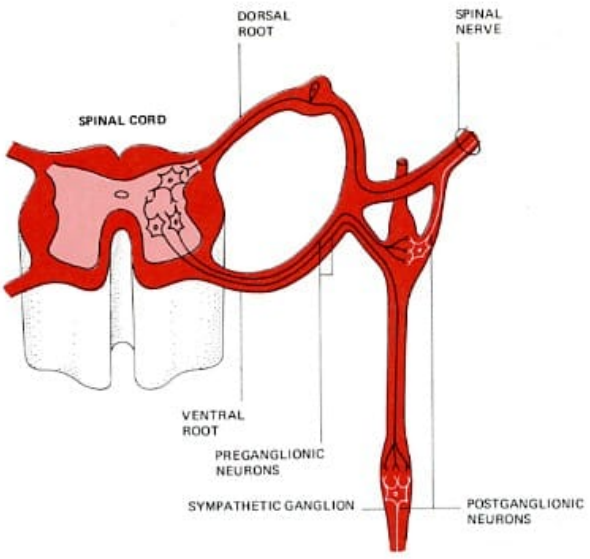


Figure 15.8.4.3 Sympathetic nervous system

The **preganglionic** motor neurons of the sympathetic system (shown in black) arise in the spinal cord. They pass into sympathetic ganglia which are organized into two chains that run parallel to and on either side of the spinal cord. The preganglionic neuron may do one of three things in the sympathetic ganglion:

- synapse with **postganglionic** neurons (shown in white) which then reenter the spinal nerve and ultimately pass out to the sweat glands and the walls of blood vessels near the surface of the body.
- pass up or down the sympathetic chain and finally synapse with postganglionic neurons in a higher or lower ganglion
- leave the ganglion by way of a cord leading to special ganglia (e.g. the solar plexus) in the viscera. Here it may synapse with postganglionic sympathetic neurons running to the smooth muscular walls of the viscera. However, some of these preganglionic neurons pass right on through this second ganglion and into the **adrenal medulla**. Here they synapse with the highly-modified postganglionic cells that make up the secretory portion of the adrenal medulla.

The neurotransmitter of the preganglionic sympathetic neurons is **acetylcholine** (**ACh**). It stimulates action potentials in the postganglionic neurons. The neurotransmitter released by the postganglionic neurons is **noradrenaline** (also called **norepinephrine**). The action of noradrenaline on a particular gland or muscle is excitatory is some cases, inhibitory in others. At excitatory terminals, ATP may be released along with noradrenaline.

The **release of noradrenaline**

- stimulates heartbeat
- raises blood pressure
- dilates the pupils
- dilates the trachea and bronchi
- stimulates glycogenolysis — the conversion of liver glycogen into glucose
- shunts blood away from the skin and viscera to the skeletal muscles, brain, and heart
- inhibits peristalsis in the gastrointestinal (GI) tract
- inhibits contraction of the bladder and rectum
- and, at least in rats and mice, increases the number of AMPA receptors in the hippocampus and thus increases long-term potentiation (LTP).

In short, stimulation of the sympathetic branch of the autonomic nervous system prepares the body for emergencies: for "**fight or flight**" (and, perhaps, enhances the memory of the event that triggered the response).

Activation of the sympathetic system is quite general because

- a single preganglionic neuron usually synapses with many postganglionic neurons

- the release of **adrenaline** from the adrenal medulla into the blood ensures that all the cells of the body will be exposed to sympathetic stimulation even if no postganglionic neurons reach them directly.

The Parasympathetic Nervous System

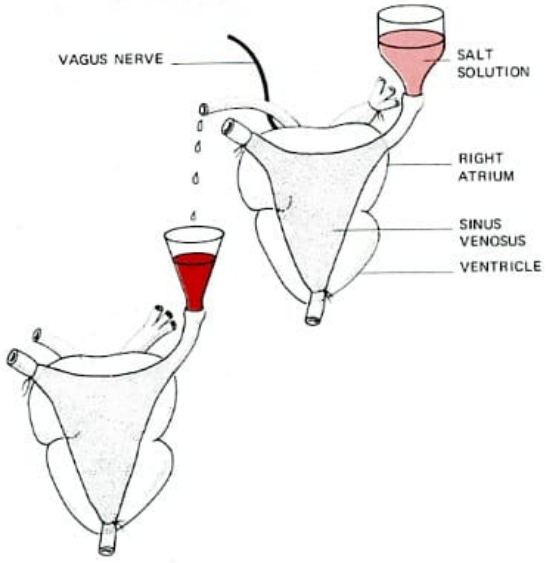


Figure 15.8.4.4 Loewi's stimulation

The Nobel Prize winning physiologist Otto Loewi discovered (in 1920) that the effect of both sympathetic and parasympathetic stimulation is mediated by released chemicals. He removed the living heart from a frog with its sympathetic and parasympathetic nerve supply intact. As expected, stimulation of the first speeded up the heart while stimulation of the second slowed it down.

Loewi found that these two responses would occur in a second frog heart supplied with a salt solution taken from the stimulated heart. Electrical stimulation of the vagus nerve leading to the first heart not only slowed its beat but, a short time later, slowed that of the second heart also. The substance responsible was later shown to be acetylcholine. During sympathetic stimulation, adrenaline (in the frog) is released.

Parasympathetic stimulation causes

- slowing down of the heartbeat (as Loewi demonstrated)
- lowering of blood pressure
- constriction of the pupils
- increased blood flow to the skin and viscera
- peristalsis of the GI tract

In short, the parasympathetic system returns the body functions to normal after they have been altered by sympathetic stimulation. In times of danger, the sympathetic system prepares the body for violent activity. The parasympathetic system reverses these changes when the danger is over. The vagus nerves also help keep inflammation under control. Inflammation stimulates nearby sensory neurons of the vagus. When these nerve impulses reach the medulla oblongata, they are relayed back along motor fibers to the inflamed area. Release of acetylcholine suppresses the release of inflammatory cytokines, e.g., tumor necrosis factor (TNF), from macrophages in the inflamed tissue.

Although the autonomic nervous system is considered to be involuntary, this is not entirely true. A certain amount of conscious control can be exerted over it as has long been demonstrated by practitioners of Yoga and Zen Buddhism. During their periods of meditation, these people are clearly able to alter a number of autonomic functions including heart rate and the rate of oxygen consumption. These changes are not simply a reflection of decreased physical activity because they exceed the amount of change occurring during sleep or hypnosis.

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15.8E: Drugs and the Nervous System

The activity of the nervous system is mediated by many kinds of interneurons releasing one or another neurotransmitter such as

- **noradrenaline**
- **gamma aminobutyric acid** (**GABA**)
- **dopamine**
- **glutamate** (Glu)
- **acetylcholine** (**ACh**)
- **serotonin**

Presynaptic neurons synthesize and package their neurotransmitter in vesicles for release (by exocytosis) at the synapse. They often have **"reuptake" transporters** that reclaim the transmitter back into the cell when it has done its job. **Postsynaptic neurons** display **receptors** to which the neurotransmitter binds. All of this machinery provides many targets for alteration by exogenous chemicals; that is, **psychoactive chemicals** introduced into the body. These drugs fall into several distinct families.

Stimulants

The most widely used stimulants are

- **caffeine** (in coffee, tea, and cola beverages)
- **nicotine** (in cigarettes)
- **amphetamines**
- **cocaine**

All of these drugs mimic the stimulation provided by the sympathetic nervous system.

Nicotine binds to a **subset** of **acetylcholine (ACh) receptors**. ACh is a neurotransmitter at synapses early in the pathways of sympathetic stimulation. Although a weak drug in one sense, nicotine is strongly addictive. The use of e-cigarettes, chewing gum and skin patches containing nicotine is designed to satisfy the craving for nicotine while avoiding the serious health effects of other ingredients in cigarette smoke.

Amphetamines and **cocaine** bind to — thus blocking — transporters used for the **reuptake of dopamine** (and noradrenaline) into presynaptic neurons. This causes the level of dopamine to rise in the synapses. **High levels of dopamine** in an area of the brain called the nucleus accumbens appear to mediate the pleasurable effects associated with these (as well as other) psychoactive drugs.

Table 1: Some amphetamines

Generic name	Trade name
dextroamphetamine sulfate	Dexedrine
methylphenidate	Ritalin
pemoline	Cylert
mixture of 4 amphetamines	Adderall

The chief medical uses for amphetamines and amphetamine-like drugs are to help people lose weight (because they suppress appetite) and to help children with attention deficit/hyperactivity disorder (**ADHD**) to perform better in school. At first glance, this second use seems counterproductive. This controversial procedure seems to work by increasing the alertness of the child so that it can focus its energies more effectively on the tasks in front of it.

Fen-Phen

Fen-Phen refers to a mixture of two amphetamine-like drugs **fenfluramine** and **phentermine** that were prescribed for losing

weight. Because of reports of occasional very serious side effects, the mixture is no longer available and fenfluramine has been removed from the U.S. market.

Cocaine

Cocaine has been used for thousands of years by certain tribes in the Andes of South America. Cocaine and some of its relatives have legitimate medical uses as local anesthetics (e.g., lidocaine). However, the widespread recreational use of cocaine has created serious social problems. In order to achieve its effects, cocaine must cross the so-called **blood-brain barrier**. If antibodies are bound to the cocaine molecule, it cannot cross. This has raised the possibility of immunizing people against cocaine. It works in mice.

Sedatives

Sedatives induce sleep. They include

- **ethanol** (beverage alcohol)
- **barbiturates**, such as
  - phenobarbital
  - secobarbital (Seconal®)
- **meprobamate** (Miltown®, Equanil®)

Ethanol

Ethyl alcohol (ethanol) is, by a wide margin, the most widely used drug in most of the world. Its popularity comes not from its sedative effect but from the sense of well-being that it induces at low doses. Perhaps low doses sedate those parts of the brain involved with, for example, tension and anxiety and in this way produce a sense of euphoria. However, higher doses depress brain centers involved in such important functions as pain sensation, coordination, and balance. At sufficiently high doses, the reticular formation can be depressed enough to cause loss of consciousness.

Ethanol increases the release of the neurotransmitter **GABA** activating **$GABA_A$** receptors and directly inhibits NMDA receptors.

Barbiturates

Barbiturates are often prescribed as sleeping pills and also to prevent seizures. Barbiturates mimic some of the action of ethanol, particularly in their ability to depress the reticular formation (thus promoting sleep) and, in high doses, the medulla oblongata (thus stopping breathing).

Barbiturates bind to a subset of GABA receptors designated **$GABA_A$** receptors. These are ligand-gated channels that enhance the flow of chloride ions (Cl^-) into the postsynaptic neuron, thus increasing its resting potential and making it less likely to fire. By binding to the $GABA_A$ receptor, barbiturates (and perhaps ethanol) increase the natural inhibitory effect of GABA synapses. Barbiturates and alcohol act additively — the combination producing a depression greater than either one alone. The combination is a frequent cause of suicide, both accidental and planned.

Meprobamate

Meprobamate is prescribed as a tranquilizer, but its action is quite different from the tranquilizers discussed below. Its molecular activity is like that of other sedatives and in combination with them can produce a lethal overdose. All sedatives produce two related physiological effects:

- **tolerance** — the necessity for a steadily-increasing dose to achieve the same physiological and psychological effects
- **physical dependence** — withdrawal of the drug precipitates unpleasant physical and psychological symptoms.

These traits are also shared with nicotine, opioids, and other psychoactive drugs.

Local Anesthetics

These chemical relatives of cocaine act by blocking the voltage-gated Na^+ channels of sensory neurons preventing them from generating **action potentials**. They are injected or applied topically and block transmission not only in pain-conducting neurons but in others as well (causing general numbness).

Examples:

- lidocaine (Xylocaine®)
- procaine (Novocaine®)

Inhaled Anesthetics

Most of these are volatile hydrocarbons or ethers. Diethyl ether and chloroform are seldom used today, having been replaced by safer alternatives such as **isofluorane**, a fluorinated ether. Some, like isofluorane, bind to inhibitory **GABA** receptors) in the brain hyperpolarizing, and thus decreasing the sensitivity of, postsynaptic neurons. Others, like ketamine, block the activity of excitatory glutamate receptors.

Other Hydrocarbons

1,4-Butanediol is a common solvent. When ingested, it is converted into γ-hydroxybutyrate, an increasingly-popular (and illegal) "club drug". γ-Hydroxybutyrate acts on $GABA_B$ receptors. Conversion of 1,4-butanediol to γ-hydroxybutyrate requires the enzyme **alcohol dehydrogenase**, the same enzyme used to metabolize ethanol. Ingesting both ethanol and 1,4-butanediol delays the effects of the latter.

Opioids

These are substances isolated from the opium poppy or synthetic relatives. (They are also called opiates.)
Examples:

- **morphine**
- **codeine**
- **heroin**
- **fentanyl** (a synthetic that is ~80 times more potent than morphine)
- **methadone**
- **oxycodone**

Opioids depress nerve transmission in sensory pathways of the spinal cord and brain that signal **pain**. This explains why opioids are such effective pain killers. Opioids also inhibit brain centers controlling coughing, breathing, and intestinal motility. Both morphine and codeine are used as pain killers, and codeine is also used in cough medicine. Opioids are exceedingly addictive, quickly producing tolerance and dependence. Although heroin is even more effective as a painkiller than morphine and codeine, it is so highly addictive that its use is illegal. **Methadone** is a synthetic opioid that is used to break addiction to heroin (and replace it with addiction to methadone).

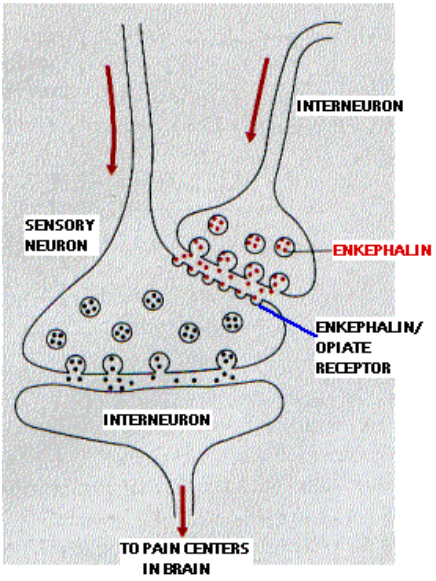


Figure 15.8.5.1 Pain signal transmission

Opioids bind to so-called **mu (μ) receptors** . These G-protein-coupled receptors are located on the subsynaptic membrane of neurons involved in the transmission of pain signals. Their **natural ligands** are two pentapeptides (containing five amino acids):

- **Met-enkephalin** (Tyr-Gly-Gly-Phe-Met-COO^-)
- **Leu-enkephalin** (Tyr-Gly-Gly-Phe-Leu-COO^-)

Release of enkephalins suppresses the transmission of pain signals. Little is to be gained by having the perception of pain increase indefinitely in proportion to the amount of damage done to the body. Beyond a certain point, it makes sense to have a system that decreases its own sensitivity in the face of massive, intractable pain.

By binding to mu (μ) receptors, opioids like morphine enhance the pain-killing effects of enkephalin neurons. Opioid tolerance can be explained, at least in part, as a homeostatic response that reduces the sensitivity of the system to compensate for continued exposure to high levels of morphine or heroin. When the drug is stopped, the system is no longer as sensitive to the soothing effects of the enkephalin neurons and the pain of withdrawal is produced.

Mu (μ) receptors are also found on the cells in the medulla oblongata that regulate breathing. This accounts for the suppressive effect opioids have on breathing.

Opioid antagonists

Opioid antagonists such as **naloxone** (Narcan®) and **naltrexone** (ReVia®) bind to μ receptors but instead of activating them, they prevent the binding of the opioids themselves. In fact, if the receptors are already occupied by, for example, heroin molecules, **naloxone** will push the heroin molecules off and quickly rescue the patient from a drug overdose. Naltrexone is used to help recovering heroin addicts stay drug-free.

Antipsychotics

Antipsychotics (also called "neuroleptics") are used to treat **schizophrenia**, a common and devastating mental disease. They act by binding to one class of receptors for the neurotransmitter **dopamine**. There are two groups currently in use:

- "Typical" antipsychotics (sometimes referred to as "major tranquilizers"). Examples:
  - **chlorpromazine** (Thorazine®)
  - **haloperidol** (Haldol®)
- "Atypical" antipsychotics (also referred to as "second generation" antipsychotics). Examples:
  - risperidone (Risperdal®)
  - olanzapine (Zyprexa®)

- quetiapine (Seroquel®)

Tranquilizers

Tranquilizers act like sedatives in reducing anxiety and tensions. Most belong to a group called **benzodiazepines** and include such commonly-prescribed drugs as Xanax® and Klonopin®. The benzodiazepines act on interneurons that use the inhibitory neurotransmitter **GABA**. By binding to $GABA_A$ receptors on the postsynaptic membrane, they enhance the action of GABA at the synapse.This is the same receptor to which barbiturates (and perhaps ethanol) bind. Thus although benzodiazepines seem safe enough when used alone, combining them with ethanol or barbiturates can be (and often has been) lethal.

Antidepressants

Antidepressants fall into four chemical categories (of which we shall examine three). Most share a common property: they increase the amount of **serotonin** at synapses that use it as a neurotransmitter.

Monoamine oxidase inhibitors (MAOIs)

These drugs act on a mitochondrial enzyme that breaks down monoamines such as **noradrenaline** and **serotonin**. By inhibiting the enzyme in presynaptic serotonin-releasing neurons, more noradrenaline and serotonin is deposited in the synapse. Some examples: Parnate®, Nardil®, Marplan®. For several reasons, MAO inhibitors are not used much anymore.

Tricyclic antidepressants (TCAs)

These drugs **block the reuptake** of **noradrenaline, dopamine, and serotonin** causing an increase in the level of these neurotransmitters in the synapse.

Examples:

Generic name	Trade name
imipramine	Tofranil®
clomiprimine	Anafranil®
amitriptyline	Elavil®

Although tricyclics are still prescribed for pain relief, their role as antidepressants has largely been taken over by the serotonin reuptake inhibitors (SRIs).

Selective serotonin reuptake inhibitors (SSRIs)

These drugs inhibit the reuptake of **serotonin** but not of noradrenaline.

Examples:

Generic name	Trade name
fluoxetine	Prozac®
paroxetine	Paxil®
sertraline	Zoloft®

Although all these drugs quickly increase the amount of serotonin in the brain, there is more to the story than that. Unlike most psychoactive drugs, antidepressants do not relieve the symptoms of depression until a week or more after dosing begins. During this period, the number of serotonin receptors on the postsynaptic membranes decreases. How this translates into relief of symptoms is not yet understood.

Serotonin and norepinephrine reuptake inhibitors (SNRIs)

Because they act on the reuptake of both serotonin and noradrenaline (norepinephrine), this category of antidepressants is also known as **dual reuptake inhibitors**.

Examples: venlafaxine (Effexor®) and duloxetine (Cymbalta®).

Bupropion

Bupropion (Wellbutrin®) is a novel drug that blocks the reuptake of **noradrenaline** and **dopamine**. Although it does not interfere with the uptake of serotonin, it also appears to be an effective antidepressant.

Atomoxetine

This drug (Strattera®) selectively interferes with the reuptake of **noradrenaline**. It is used in children with attention deficit/hyperactivity disorder (ADHD).

Psychedelics

Psychedelic drugs distort sensory perceptions, especially sight and sound. Some such as mescaline, psilocybin and dimethyltryptamine (DMT) are natural plant products.



Figure 15.8.5.2 Peyote cactus courtesy of Dr. Richard Evans Schultes

The photograph shows the peyote cactus in flower. The cactus head contains several psychedelic chemicals, of which mescaline is the most important. Dried cactus heads ("mescal buttons") have been used since pre-Columbian times in the religious ceremonies of native peoples in Mexico. About a century ago, this religious use spread to some tribes in the United States and Canada who, in 1922, became incorporated into the Native American Church. Other psychedelic drugs are synthetic. These include

- lysergic acid diethylamide (**LSD**)
- dimethoxymethylamphetamine (DOM or "STP")
- methylenedioxymethamphetamine (MDMA or "ecstasy")

As their name suggests, DOM and MDMA also share the stimulant qualities of amphetamines. All the psychedelics have a molecular structure that resembles serotonin and probably bind to **serotonin receptors** on the postsynaptic membrane.

Phencyclidine (PCP)

PCP is used as an anesthetic in veterinary medicine. Used (illicitly) by humans (called "crystal" or "angel dust"), it can produce a wide variety of powerful reactions resembling those of stimulants as well as psychedelics. Unlike the other psychedelics, it binds to (and inhibits) NMDA receptors (in the hippocampus and other parts of the forebrain).

Marijuana

The main psychoactive ingredient in marijuana is **delta-9-tetrahydrocannabinol** (Δ^9-**THC**). It binds to

- **CB_1 receptors** (G-protein-coupled receptors) that are present on presynaptic membranes in many parts of the brain.
- CB_2 receptors are also found in the brain as well as being highly-expressed on cells of the immune system (e.g., B cells and T cells).

THC produces the drowsiness of sedatives like alcohol, the dulling of pain (like opioids) and in high doses, the perception-distorting effects of the psychedelics. Unlike sedatives and opioids, however, tolerance to THC does not occur. In fact, the drug is excreted so slowly from the body that, with repeated use, a given response is achieved by a lower dose.

The natural ligands of the CB receptors are the endocannabinoids - **anandamide** and **2-arachidonylglycerol** (2-AG). Both of these compounds are produced from phospholipids.

What are these natural ligands doing? They probably will turn out to have multiple effects, but the clearest ones so far are their effects on

- **appetite**. Mice given **anandamide** eat more than normal while those whose genes for the CB_1 receptor have been "knocked out" eat less than normal.These findings will be no surprise to the ill humans (e.g., with cancer or AIDS) who find that marijuana improves their appetite. Rimonabant (Acomplia®), a drug that blocks the ability of the body's natural CB_1 ligands to bind the CB_1 receptor was sold for a time in Europe as an **appetite suppressant**. (Because of its side effects, it was never approved for use in the U.S. and was removed from the European market in 2008.)
- **development of correct synaptic connections in the embryonic brain**. Mice whose genes for the CB_1 receptor have been knocked out develop defects in the wiring pattern of interneurons in their brain (which may account for the cognitive defects that have been reported in the children of women who used marijuana during pregnancy).
- **neuronal activity in the adult brain**. Mice whose genes for the CB_1 receptor have been knocked out are more susceptible to epileptic seizures. Marijuana has been used for centuries to control epileptic seizures in humans.
- **suppressing contact dermatitis**. Knockout mice lacking CB_1 and CB_2 receptors mount a more vigorous allergic inflammatory response to agents (like nickel) that elicit contact sensitivity.

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15.8F: Nitric Oxide (NO)

Nitric oxide is a gas. It is highly reactive; that is, it participates in many chemical reactions. (It is one of the nitrogen oxides ("NO_X") in automobile exhaust and plays a major role in the formation of **photochemical smog.**) But NO also has many physiological functions.

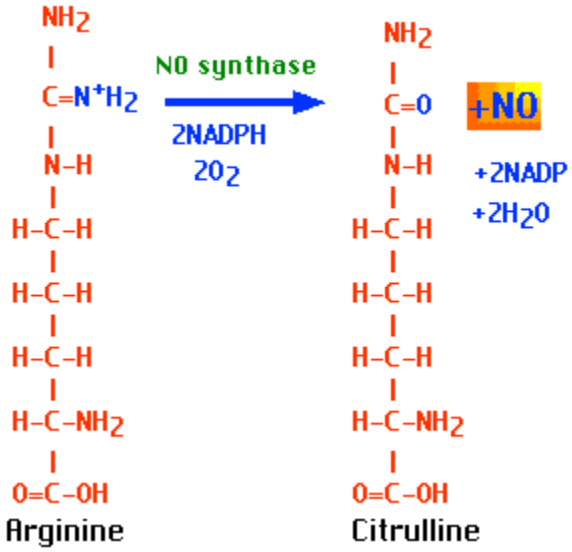


Figure 15.8.6.1 NO Synthase

They share these features:

- NO is synthesized within cells by an enzyme **NO synthase** (**NOS**).
- The human (and mouse) genome contains 3 different genes encoding NO synthases.
  - **nNOS** (or NOS-1): found in neurons (hence the "n").
  - **eNOS** (or NOS-3): found in the endothelial (hence the "e") cells that line the lumen of blood vessels.
  - **iNOS** (or NOS-2): found in macrophages. (the "i" stands for "inducible"). Whereas the levels of nNOS and eNOS are relatively steady, expression of iNOS genes awaits an appropriate stimulus (e.g., invasion by a pathogen).
- All types of NOS produce NO from arginine with the aid of molecular oxygen and NADPH.
- NO diffuses freely across cell membranes.
- There are so many other molecules with which it can interact, that it is quickly consumed close to where it is synthesized.
- Thus NO acts in a paracrine or even autocrine fashion - affecting only cells near its point of synthesis.

This page examines some of the functions of NO.

Blood Flow

NO relaxes the smooth muscle in the walls of the arterioles. At each systole, the endothelial cells that line the blood vessels release a puff of NO. This diffuses into the underlying smooth muscle cells causing them to relax and thus permit the surge of blood to pass through easily. Mice whose genes for the NO synthase found in endothelial cells (eNOS) has been "knocked out" suffer from hypertension.

Nitroglycerine, which is often prescribed to reduce the pain of angina, does so by generating nitric oxide, which relaxes the walls of the coronary arteries and arterioles. NO also inhibits the aggregation of platelets and thus keeps inappropriate clotting from interfering with blood flow.

Kidney Function

Release of NO around the glomeruli of the kidneys increases blood flow through them thus increasing the rate of filtration and urine formation.

Penile Erection

The erection of the penis during sexual excitation is mediated by NO released from nerve endings close to the blood vessels of the penis. Relaxation of these vessels causes blood to pool in the blood sinuses producing an erection. Three popular prescription drugs enhance this effect by the mechanism described below.

Recent evidence suggests that NO's job in reproduction is not finished with producing an erection. At the moment of contact, release of NO by the acrosome of the sperm activates the egg to complete meiosis II and the other steps of fertilization.

Other Actions on Smooth Muscle

Peristalsis

The wavelike motions of the gastrointestinal tract are aided by the relaxing effect of NO on the smooth muscle in its walls.

Birth

NO also inhibits the contractility of the smooth muscle wall of the uterus. As the moment of birth approaches, the production of NO decreases. Nitroglycerine has helped some women who were at risk of giving birth prematurely to carry their baby to full term.

NO and Inflammation

The NO produced by eNOS (NOS-3) inhibits inflammation in blood vessels. It does this by blocking the exocytosis of mediators of inflammation from the endothelial cells. NO may also block exocytosis in other types of cells such as macrophages and cytotoxic T lymphocytes (CTL).

Effects on Secretion

NO affects secretion from several endocrine glands.

For examples, it stimulates

- the release of Gonadotropin-releasing hormone (GnRH) from the hypothalamus
- the release of pancreatic amylase from the exocrine portion of the pancreas
- the release of adrenaline from the adrenal medulla

NO and the Nervous System

NO and the Autonomic Nervous System

Some motor neurons of the parasympathetic branch of the autonomic nervous system release NO as their neurotransmitter. The actions of NO on penile erection and peristalsis are probably mediated by these nerves.

NO and the Medulla Oblongata

Hemoglobin transports NO at the same time it carries oxygen. When it unloads oxygen in the tissues, it also unloads NO. In severe deoxygenation, NO-sensitive cells in the medulla oblongata respond to this release by increasing the rate and depth of breathing.

NO and the Brain

In laboratory animals (mice and rats), NO is released by neurons in the CA1 region of the hippocampus and stimulates the NMDA receptors there that are responsible for long-term potentiation (LTP) - a type of memory (and learning). The ease with which NO diffuses away from the synapse where it is generated enables it to affect nearby synapses. So what may have begun as a localized action becomes magnified.

Laboratory rats treated with inhibitors of NOS synthesis fail to develop and/or retain learned responses such as the conditioned response. Mice whose genes for nNOS have been knocked out are healthy but display abnormal behavior, e.g., they kill other males and try to mate with nonreceptive females.

NO and Fertilization

The acrosome at the tip of sperm heads activates its NO synthase when it enters the egg. The resulting release of NO in the egg is essential (at least in sea urchins) for triggering the next steps in the process :

- blocking the entry of additional sperm
- orienting the pronuclei for fusion.

Killing Pathogens

NO aids in the killing of engulfed pathogens (e.g., bacteria) within the lysosomes of macrophages.

Mice whose genes for the NO synthase found in macrophages (iNOS) have been knocked out are more susceptible to infections by intracellular bacteria like *Listeria monocytogenes*. Th1 cells, the ones responsible for an inflammatory response against invaders, secrete NO. Harmless bacteria, living as commensals at the rear of our throat, convert nitrates in our food into nitrites. When these reach the stomach, the acidic gastric juice (pH ~1.4) generates NO from them. This NO kills almost all the bacteria that have been swallowed in our food.

Since the dawn of recorded human history, nitrites have been used to preserve meat from bacterial spoilage.

NO and Longevity

Mice whose genes for eNos have been knocked out

- show signs of premature aging
- have a shortened life span
- fail to benefit from the life-extending effect of a calorie-restricted (CR) diet

Mechanisms of NO Action

The signaling functions of NO begin with its binding to protein receptors on or in the cell. The binding sites can be either:In either case, binding triggers an allosteric change in the protein which, in turn, triggers the formation of a "second messenger" within the cell. The most common protein target for NO seems to be **guanylyl cyclase**, the enzyme that generates the second messenger **cyclic GMP** (**cGMP**).

Three prescription drugs sildenafil (Viagra®), vardenafil (Levitra®) and tadalafil (Cialis®) enhance the effects of NO by inhibiting the enzyme that normally breaks down cGMP.

Fireflies Use NO To Turn On their Flashes

Bioluminescence

Plants Also Use NO

NO has been implicated in many plant activities. It is a weapon against invading pathogens. Infection of the plant triggers the formation of a NOS that, like the animal versions, makes NO from arginine. Release of NO by the infected cell induces a number of defense responses. A gradient of NO may also guide the pollen tube to its destination in the ovule. The popular prescription drug sildenafil citrate (Viagra®) enhances the effect of NO on pollination (just as it does on penile erection). NO inhibits flowering. NO promotes recovery from etiolation.

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15.8G: Prion Diseases

Prion diseases are transmissible from host to host of a single species and, sometimes, even from one species to another (such as a laboratory animal). They destroy brain tissue giving it a spongy appearance. For these reasons, prion diseases are also called **transmissible spongiform encephalopathies** or **TSE**s.

Some examples:

Creutzfeldt-Jakob Disease	CJD	humans
variant Creutzfeldt-Jakob Disease	vCJD	humans; acquired from cattle with BSE
Bovine Spongiform Encephalopathy	BSE	"mad cow disease"
Kuru		infectious; in humans who practiced cannibalism in Papua New Guinea
Gerstmann-Sträussler-Scheinker disease	GSS	inherited disease of humans
Fatal Familial Insomnia	FFI	inherited disease of humans
Scrapie		infectious disease of sheep and goats
other animal TSEs		cats, mink, elk, mule deer

Before the victim dies of a TSE, the damage to the brain is reflected in such signs as loss of coordination and in humans dementia. Injections of ground-up brain tissue from an animal or human patient with a prion disease into another animal (of the appropriate species) transmits the disease. This suggests that the disease is caused by an infectious agent such as a virus. But viruses have a genome and despite intense efforts no evidence of a virus has ever been found in these brain extracts. In fact, treating the extracts with agents (e.g., ultraviolet light) that destroy DNA does not reduce their infectiousness.

To date, the evidence indicates that the infectious agent in the TSEs is a protein. Stanley Prusiner who pioneered in the study of these proteins and was awarded the Nobel Prize in 1997 for his efforts has named them prion proteins (designated **PrP**) or simply **prions**. It turns out that prions are molecules of a normal body protein that have **changed their three-dimensional configuration**.

PrP^C

The normal protein

- is called **PrP^C** (for cellular)
- is a glycoprotein normally anchored to the surface of cells.
- has its secondary structure dominated by alpha helices (probably 3 of them)
- is easily soluble
- is easily digested by proteases
- is encoded by a gene designated (in humans) ***PRNP*** located on our chromosome 20.

PrP^{Sc}

The abnormal, disease-producing protein

- is called **PrP^{Sc}** (for scrapie)
- has the same amino acid sequence as the normal protein; that is, **their primary structures are identical** but
- its secondary structure is dominated by beta conformation
- is insoluble in all but the strongest solvents
- is highly resistant to digestion by proteases
- When PrP^{Sc} comes in contact with PrP^C, it converts the PrP^C into more of itself (even in the test tube).

- These molecules bind to each other forming aggregates.
- It is not yet clear if these aggregates are themselves the cause of the cell damage or are simply a side effect of the underlying disease process.

Inherited Prion Diseases

Creutzfeldt-Jakob Disease (CJD)

10–15% of the cases of CJD are inherited; that is, the patient comes from a family in which the disease has appeared before. The disease is inherited as an autosomal **dominant**. The patients have inherited at least one copy of a mutated *PRNP* gene. Some of the most common mutations are:

- a change in codon 200 converting glutamic acid (E) at that position to lysine (K) (thus designated "E200K")
- a change from aspartic acid (D) at position 178 in the protein to asparagine (D178N) when it is accompanied by a polymorphism in both *PRNP* genes that encodes valine at position 129. When the polymorphism at codon 129 is Met on both genes, the D178N mutation produces Fatal Familial Insomnia instead.
- a change from valine (V) at position at position 210 to isoleucine (V210I)

Extracts of autopsied brain tissue from these patients can transmit the disease to

- apes (whose *PRNP* gene is probably almost identical to that of humans).
- transgenic mice who have been given a *Prnp* gene that contains part of the human sequence.

These results lead to the important realization that prion diseases can only be transmitted to animals that already carry a *PRNP* gene with a sequence that is at least similar to the one that encoded the **PrP^{Sc}**. In fact, knockout mice with no *Prnp* genes at all cannot be infected by PrP^{Sc}.

Gerstmann-Sträussler-Scheinker disease (GSS)

This prion disease is caused by the inheritance of a *PRNP* gene with a mutations encoding most commonly

- leucine instead of proline at position 102 (P102L) or
- valine instead of alanine at position 117 (A117V)

Again, the disease is also strongly associated with homozygosity for a polymorphism at position 129 (both residues being methionine).

Brain extracts from patients with GSS can transmit the disease to

- monkeys and apes
- transgenic mice containing a portion of the human *PRNP* gene.

Transgenic mice expressing the P102L gene develop the disease spontaneously.

Fatal Familial Insomnia (FFI)

People with this rare disorder have inherited

- a *PRNP* gene with asparagine instead of aspartic acid encoded at position 178 (D178N)
- the susceptibility polymorphism of methionine at position 129 of the *PRNP* genes.

Extracts from autopsied brains of FFI victims can transmit the disease to transgenic mice.

Infectious Prion Diseases

Kuru

Kuru was once found among the Fore tribe in Papua New Guinea whose rituals included eating the brain tissue of recently deceased members of the tribe. Since this practice was halted, the disease has disappeared. Before then, the disease was studied by transmitting it to chimpanzees using injections of autopsied brain tissue from human victims.

Scrapie

This disease of sheep (and goats) was the first TSE to be studied. It seems to be transmitted from animal to animal in feed contaminated with nerve tissue. It can also be transmitted by injection of brain tissue.

Bovine Spongiform Encephalopathy (BSE) or "Mad Cow Disease"

An epidemic of this disease began in Great Britain in 1985 and before it was controlled, some 800,000 cattle were sickened by it. Its origin appears to have been cattle feed that contained brain tissue from sheep infected with scrapie and had been treated in a new way that no longer destroyed the infectiousness of the scrapie prions.

The use of such food was banned in 1988 and after peaking in 1992, the epidemic declined quickly.

Creutzfeldt-Jakob Disease (CJD)

A number of humans have acquired CJD through accidental exposure to material contaminated with CJD prions.

- Grafts of dura mater taken from patients with inherited CJD have transmitted the disease to 228 recipients.
- Corneal transplants have also inadvertently transmitted CJD.
- Instruments used in brain surgery on patients with CJD have transmitted the disease to other patients. Two years after their supposed sterilization, these instruments remained infectious.
- 226 people have acquired CJD from injections of human growth hormone (HGH) or human gonadotropins prepared from pooled pituitary glands that inadvertently included glands taken from humans with CJD.

Now that both HGH and human gonadotropins are available through recombinant DNA technology, such disastrous accidents need never recur.

Variant Creutzfeldt-Jakob Disease (vCJD)

This human disorder appeared some years after the epidemic of BSE (Mad Cow Disease) swept through the cattle herds in Great Britain. Even though the cow and human *PRNP* genes differ at 30 codons, the sequence of their prions suggests that these patients (155 by 2005) acquired the disease from eating contaminated beef.

All the patients are homozygous for the susceptibility polymorphism of methionine at position 129. The BSE epidemic has waned, and slaughter techniques that allow cattle nervous tissue in beef for human consumption have been banned since 1989. Now we must wait to see whether more cases of vCJD are going to emerge or whether the danger is over.

Miscellaneous Infectious Prion Diseases

A number of TSEs have been found in other animals. Cats are susceptible to Feline Spongiform Encephalopathy (FSE). Mink are also susceptible to a TSE. Even though mad cow disease has not been seen in North America, a similar disease is found in elk and mule deer in the Rocky Mountains of the U.S.

Sporadic Prion Diseases

CJD and FFI occasionally occur in people who have no family history of the disease and no known exposure to infectious prions.

The cause of their disease is uncertain.

- Perhaps a spontaneous somatic mutation has occurred in one of the *PRNP* genes in a cell.
- Perhaps their normal PrP^{C} protein has spontaneously converted into the PrP^{Sc} form.
- Or perhaps the victims were simply unknowingly exposed to infectious prions, and sporadic prion diseases do not exist!

Whatever the answer, all the cases are found in people with a susceptibility polymorphism in their *PRNP* genes.

Prions in Yeast

Two proteins in yeast (***Saccharomyces cerevisiae***)

- the Sup35 protein ("Sup35p") and
- the Ure2 protein (Ure2p)

are able to form prions; that is, they can exist either

- in a **PrP^{C}**-like form that is functional or
- in a **PrP^{Sc}**-like form that is not.

The greater ease with which yeast can be studied has proved that only protein is involved in prion formation and provided insight into the need for **PrP^{Sc}** to find **PrP^{C}** molecules of a similar primary structure in order to be able to convert them into the **PrP^{Sc}** form.

Evidence that prions are a "protein-only" phenomenon

- A few molecules of a **PrP^{Sc}** form of the Sup35 protein, when introduced into yeast cells, convert the yeast cell's own Sup35 protein into prion aggregates. The resulting "disease" phenotype is then passed on to the cell's daughters.

  The introduced protein was synthesized in bacteria making it unlikely that it could be contaminated by any gene-containing infectious agent of yeast.

- Yeast can be "cured" of their prion "disease" by increasing the activity of their chaperones. Presumably the chaperone helps maintain the folded state (with alpha helices) of the protein.
- When the gene for the glucocorticoid receptor is altered to include sequences coding for one part (domain) of the Sup35 protein, the resulting protein forms prions and produces an entirely new phenotype.

Possible basis of species specificity of prions

- A particular **PrP^{Sc}** can only convert **PrP^{C}** molecules of the same or at least similar primary structure.
- This requirement of "like-with-like" resides in a short sequence at the N-terminal of the protein (rather like an antibody epitope).
- Yeasts engineered to form two types of prion form two types of "pure" aggregates within the cell.
- Even in the test tube, each type of prion finds and aggregates with others of its own type.

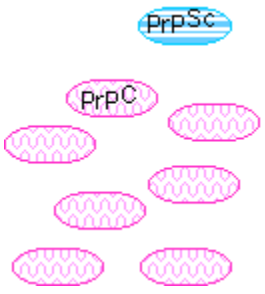


Figure 15.8.7.1 PrP

So the picture that emerges is that a molecule of **PrP^{Sc}** acts as a "seed" providing a template for converting **PrP^{C}** to more **PrP^{Sc}**. These interact with each other to form small soluble aggregates. These interact with each other to form large insoluble deposits. Although only a small portion of the prion protein is responsible for its specificity, other parts of the molecule are needed for flipping the molecule from the alpha-helical to the beta conformation. All prion proteins contain tracts of repeated **Gln-Asn** residues which appear to be essential for the conversion process.

Other Pathogenic Prion-like Proteins

The deposits of **PrP^{Sc}** in the brain are called **amyloid**. Amyloid deposits are also found in other diseases.

Examples:

- Alzheimer's disease is characterized by amyloid deposits of
  - the peptide amyloid-beta (Aβ)
  - the protein tau

  in the brain.
- The brains of Parkinson's disease patients have deposits of α-synuclein.
- Deposits of the protein **huntingtin** are found in the brains of victims of Huntington's disease.
- Amyloid deposits of the protein transthyretin are found in peripheral nerves, the kidney, and other organs.

With all of these diseases there is evidence that their amyloid-forming proteins, like PrP^{Sc}, can act as a "seed" converting a correctly-folded protein into an incorrectly-folded one and have this effect spread from cell to cell. However, they do not seem to be able to be spread from person to person (unlike the TSEs). Perhaps this is because they are not so incredibly resistant to degradation as PrP^{Sc} is.

Most cells, including neurons in the brain, contain **proteasomes** that are responsible for degrading misfolded or aggregated proteins. In the various brain diseases characterized by a build-up of amyloid deposits, it appears that as the small insoluble amyloid precursors accumulate, they bind to proteasomes but cannot be degraded by them. Furthermore, this binding blocks the ability of the proteasomes to process other proteins that are normal candidates for destruction. Because of the critical role of proteasomes in many cell functions, such as mitosis, it is easy to see why this action leads to death of the cell.

Prion-like proteins not always harmful

Evidence:

- Yeast are not harmed when Sup35p and Ure2p form prions.
- The role of CPEB.

CPEB ("**c**ytoplasmic **p**olyadenylation **e**lement **b**inding protein") is a protein that

- is found in neurons of the central nervous system (as well as elsewhere)
- stimulates messenger RNA (mRNA) translation
- is needed for long-term facilitation (**LTF**)
- accumulates at activated (by serotonin) synapses
- has the ability to undergo a change in tertiary structure that
  - persists for long periods
  - induces the same conformational change in other molecules of CPEB forming prion-like aggregates

Perhaps the accumulation of these aggregates at a stimulated synapse causes a long-term change in its activity (memory).

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15.9: SENSES

15.9A: Mechanoreceptors
15.9B: Hearing
15.9C: Vision
15.9D: Processing Visual Information
15.9E: Vision in Arthropods
15.9F: Heat, Cold, and Pain Receptors
15.9G: Taste
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15.9I: Electric Organs and Electroreceptors
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15.9A: Mechanoreceptors

We and other animals have several types of receptors of mechanical stimuli. Each initiates nerve impulses in sensory neurons when it is physically deformed by an outside force such as: touch, pressure, stretching, sound waves, and motion. Mechanoreceptors enable us to detect touch, monitor the position of our muscles, bones, and joints — the sense of proprioception and detect sounds and the motion of the body.

Touch

Light touch is detected by receptors in the skin. Many of these are found next to hair follicles so even if the skin is not touched directly, movement of the hair is detected. Touch receptors are not distributed evenly over the body. The fingertips and tongue may have as many as 100 per cm^2; the back of the hand fewer than 10 per cm^2. This can be demonstrated with the **two-point threshold test**. With a pair of dividers like those used in mechanical drawing, determine (in a blindfolded subject) the minimum separation of the points that produces two separate touch sensations. The ability to discriminate the two points is far better on the fingertips than on, say, the small of the back. The density of touch receptors is also reflected in the amount of somatosensory cortex in the brain assigned to that region of the body.

Proprioception

Proprioception is our "body sense". It enables us to unconsciously monitor the position of our body. It depends on receptors in the muscles, tendons, and joints. If you have ever tried to walk after one of your legs has "gone to sleep," you will have some appreciation of how difficult coordinated muscular activity would be without proprioception.

Four Mechanoreceptors

1: The Pacinian Corpuscle

Pacinian corpuscles are pressure receptors. They are located in the skin and also in various internal organs. Each is connected to a sensory neuron. Because of its relatively large size, a single Pacinian corpuscle can be isolated and its properties studied. Mechanical pressure of varying strength and frequency is applied to the corpuscle by the stylus. The electrical activity is detected by electrodes attached to the preparation.

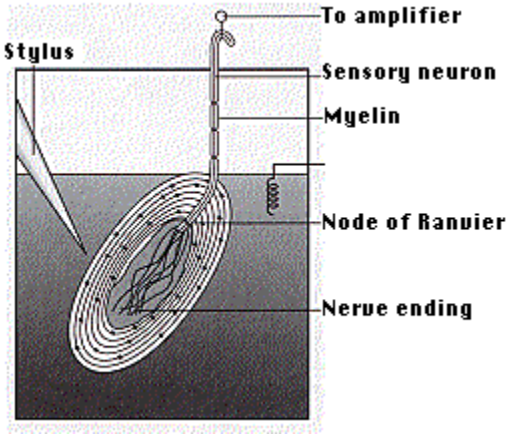


Figure 15.9.1.1 Pacinian

Deforming the corpuscle creates a generator potential in the sensory neuron arising within it. This is a graded response: the greater the deformation, the greater the generator potential. If the generator potential reaches threshold, a volley of action potentials (also called nerve impulses) are triggered at the first node of Ranvier of the sensory neuron. Once threshold is reached, the magnitude of the stimulus is encoded in the **frequency** of impulses generated in the neuron. So the more massive or rapid

the deformation of a single corpuscle, the higher the frequency of nerve impulses generated in its neuron.

2: Adaptation

When pressure is first applied to the corpuscle, it initiates a volley of impulses in its sensory neuron. However, with **continuous** pressure, the frequency of action potentials decreases quickly and soon stops. This is the phenomenon of adaptation. Adaptation occurs in most sense receptors. It is useful because it prevents the nervous system from being bombarded with information about insignificant matters like the touch and pressure of our clothing. Stimuli represent **changes** in the environment. If there is no change, the sense receptors soon adapt. But note that if we quickly remove the pressure from an adapted Pacinian corpuscle, a fresh volley of impulses will be generated. This is why Pacinian corpuscles respond especially well to vibrations.

The speed of adaptation varies among different kinds of receptors. Receptors involved in proprioception such as spindle fibers adapt slowly if at all.

3: Meissner Corpuscles

Meissner corpuscles, like Pacinian corpuscles, adapt quickly to a sustained stimulus but are activated again when the stimulus is removed. Thus they are especially sensitive to movement across the skin.

4: Merkel Cells

Merkel cells are transducers of light touch, responding to the texture and shape of objects indenting the skin. Unlike Pacinian and Meissner corpuscles, they do not adapt rapidly to a sustained stimulus; that is, they continue to generate nerve impulses so long as the stimulus remains. They are found in the skin often close to hairs. They form synapses with Aβ sensory neurons leading back to the CNS.

In the rat, light movement of a hair triggers a generator potential in a Merkel cell. If this reaches threshold, an influx of Ca^{++} ions through voltage-gated calcium channels generate action potentials in the Merkel cell. These cause the release of neurotransmitters at the synapse with its Aβ sensory neuron. (This neuron may also have its own mechanically-gated ion channels able to directly generate action potentials more rapidly than Merkel cells can.)

The knee jerk Reflex

The knee jerk is a stretch reflex. Your physician taps you just below the knee with a rubber-headed hammer. You respond with an involuntary kick of the lower leg.

- The hammer strikes a tendon that inserts an extensor muscle in the front of the thigh into the lower leg.
- Tapping the tendon stretches the thigh muscle.
- This activates stretch receptors within the muscle called muscle spindles. Each **muscle spindle** consists of
  - sensory nerve endings wrapped around
  - special muscle fibers called **spindle fibers** (also called intrafusal fibers)
- Stretching a spindle fiber initiates a volley of impulses in the sensory neuron (called an "**I-a**" neuron) attached to it.
- The impulses travel along the sensory axon to the spinal cord where they form several kinds of synapses:
  - Some of the branches of the I-a axons synapse directly with **alpha motor neurons** (Pacinian Corpuscle). These carry impulses back to the same muscle causing it to contract. The leg straightens.
  - Some of the branches of the I-a axons synapse with **inhibitory interneurons** in the spinal cord (Meissner Corpuscles). These, in turn, synapse with motor neurons leading back to the **antagonistic muscle**, a **flexor** in the back of the thigh. By inhibiting the flexor, these interneurons aid contraction of the extensor.
  - Still other branches of the I-a axons synapse with interneurons leading to brain centers, e.g., the cerebellum, that coordinate body movements (Merkel Cells).

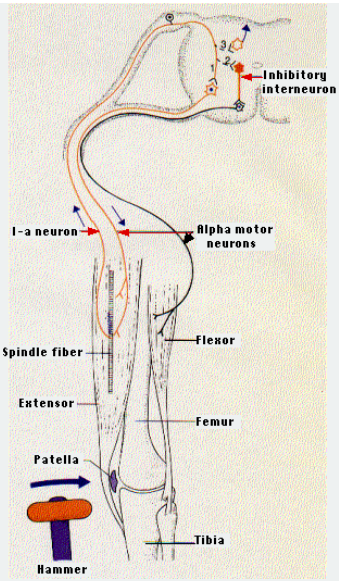


Figure 15.9.1.2 Kneejerk reflex

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15.9B: Hearing

The sense of hearing is the ability to detect the mechanical vibrations we call sound. Sound waves pass down the auditory canal of the outer ear and strike the eardrum (tympanic membrane) causing it to vibrate. These vibrations are transmitted across the **middle ear** by three tiny linked bones, the **ossicles**:

- hammer (malleus)
- anvil (incus)
- stirrup (stapes)
- The ossicles also magnify the amplitude of the vibrations.

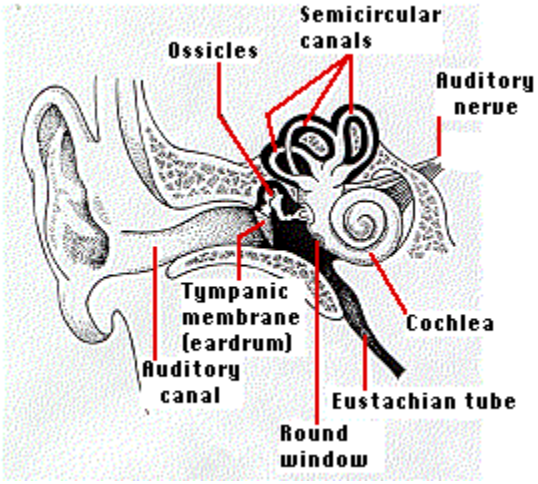


Figure 15.9.2.1 Ear

The middle ear is filled with air and is connected to the outside air by the **eustachian tube**, which opens into the nasopharynx. Opening of the tube during swallowing or yawning equalizes the air pressure on either side of the eardrum. Allergies or a head cold may inflame the walls of the eustachian tubes making them less easily opened. Rapid changes in pressure at such times — such as descending in an aircraft or during a SCUBA dive, may be quite painful because of the unequal pressure against the eardrums.

The Inner Ear

Vibrations of the innermost ossicle, the stirrup, are transmitted through a flexible membrane, the **oval window** to the **cochlea** of the inner ear. The cochlea is a tube, about 3.5 cm long, that is coiled like a snail shell and filled with a special fluid called **endolymph**. The most dramatic difference in the composition of endolymph from other lymph in the body is its high concentration of potassium (K^+) ions. Running through the cochlea for its entire length is a plate of bone and an inner tube that is also filled with endolymph. These structures divide the outer tube of the cochlea into two separate chambers.

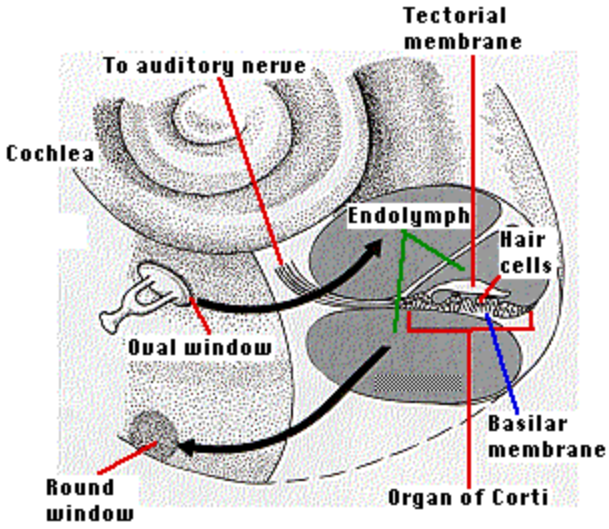


Figure 15.9.2.2 Inner ear

Because liquids are practically incompressible, it is necessary to have some way of relieving the pressures created when the oval window is pushed in and out. The flexible **round window** does this by moving in the opposite direction.

The organ of Corti

The organ of Corti lies within the middle chamber of the cochlea. It contains thousands of **hair cells**, which are the actual vibration receptors. The apical surface of the hair cells contains an array of **stereocilia**, which give the hair cells their name. Stereocilia are not built from the "9+2" arrangement of microtubules that are found in true cilia. The hair cells are located between the **basilar** and **tectorial membranes**. Vibrations of the endolymph cause vibrations of the basilar membrane. This moves stereocilia at the tips of the hair cells against the tectorial membrane and open potassium channels in them. The influx of K^+ from the endolymph depolarizes the cell.

You should note that hair cells differ from most "excitable cells" (neurons and muscle fibers) in their use of potassium ions, not sodium ions, to depolarize the cell. Depolarization of the hair cell causes the release of a neurotransmitter (probably glutamate) at its basal surface and the initiation of nerve impulses in a sensory neuron that synapses with it. These impulses travel back along the **auditory nerve** (the 8th cranial nerve) to the brain.

Many people, especially when young, can hear sounds with frequencies (pitches) from as low as 16 to as high as 20,000 **hertz** (cycles per second). Detection of a given frequency is a function of the location of the hair cells along the organ of Corti with the highest frequencies detected near the base of the cochlea, and the remainder of the sound spectrum detected in a progressive fashion with the lowest frequencies detected by hair cells near the tip.

Deafness

Deafness may be acquired or inherited.

Acquired deafness

If a laboratory animal is exposed to very intense, pure tones, it eventually becomes deaf to those frequencies, but its ability to hear other pitches is unimpaired. Examination of its organ of Corti reveals destroyed hair cells in a single area whose location can be easily correlated with the pitch of the destructive sound. Similar deficits occur in humans who are exposed to intense noises for long periods. A trained audiologist can tell by looking at the frequency response whether a patient flies private aircraft.

Inherited deafness

About 1 newborn in a thousand is born deaf because of a genetic defect. As the years go by, many of us (~16%) suffer a

progressive loss of hearing because of genetic defects. Literally scores of genes have been identified in recent years whose mutant versions result in hearing loss.

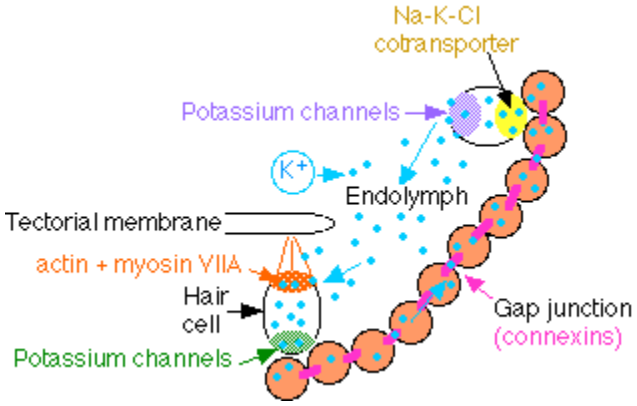


Figure 15.9.2.3 K - cycle

Mutations in a transcription factor have been associated with a stirrup (stapes) that cannot move freely and thus cannot transmit vibrations to the oval window. The proper organization of the stereocilia involves **actin**, a form of **myosin** (called myosin VIIA), and **cadherins**.also cause deafness. Mutations in the gene encoding a protein that helps with actin polymerization cause deafness. Mutations in the myosin VIIA gene and mutations in the gene encoding cadherin 23.

The potassium that enters the hair cells must be removed from them and recycled back to the endolymph for hearing to continue. Scores of mutations in the necessary transport molecules have been linked to inherited deafness. Mutations in genes encoding the K^+ channels that allows K^+ to leave the hair cell through its basolateral surface (shown in green). (These same channels are found in the loops of Henle in the kidneys so the mutations can produce defects in kidney function as well as deafness.) Mutations in the connexins (magenta) that form the gap junctions through which the K^+ passes from cell to cell on its way back to the secretory cells that will deposit it back in the endolymph. Mutations in the sodium-potassium-chloride cotransporter (shown in yellow) that actively transports K^+ against its concentration gradient into the secretory cells. Mutations in the K^+ channels (shown in lavender) that allow for the facilitated diffusion of K^+ out of the secretory cells and into the endolymph.

Equilibrium

The inner ear also detects:

- the position of the body with respect to gravity
- the motion of the body.

Just above the cochlea are two interconnecting chambers filled with endolymph, the **sacculus** and **utriculus**. On their inner surface are patches of **hair cells** to which are attached thousands of tiny spheres of calcium carbonate ($CaCO_3$). Gravity pulls these downward. As the head is oriented in different directions, these **ear stones** or **otoliths** shift their position. The action potentials initiated in the hair cells are sent back to the brain.

Motion of the body is detected in the three **semicircular canals** at the top of each inner ear, each one oriented in a different plane. There is a small chamber at one end of each canal containing hair cells. Whenever the head is moved, the fluid within the canals lags in its motion so that there is relative motion between the walls and the endolymph. This stimulates the hair cells to send impulses back to the brain.

When the hair cells send messages that are incongruent with what the eyes are seeing and our body is feeling, as may occur in a boat or aircraft during rough weather, motion sickness can result. Some people also suffer severe dizziness because otoliths have become dislodged from their utriculus (e.g. following a blow to the head) and settled in a semicircular canal.

Echolocation

Bats can hear frequencies as high as 150,000 hertz. Sound at these ultrasonic (to us) frequencies travels in fairly straight lines. Bats flying in complete darkness are able to locate obstacles and even insect prey by emitting pulses of this ultrasonic sound and then adjusting the course of flight to the echo that returns to their ears. Such a system of echolocation works on the same principle as sonar for submarine detection.

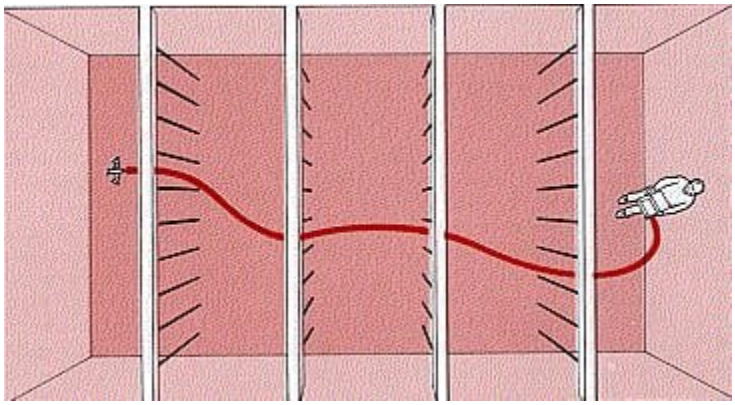


Figure 15.9.2.4 Echolocation of a blindfolded bat

A blindfolded bat can fly between the wires touching them only rarely. A bat whose ears are plugged collides repeatedly with the wires.

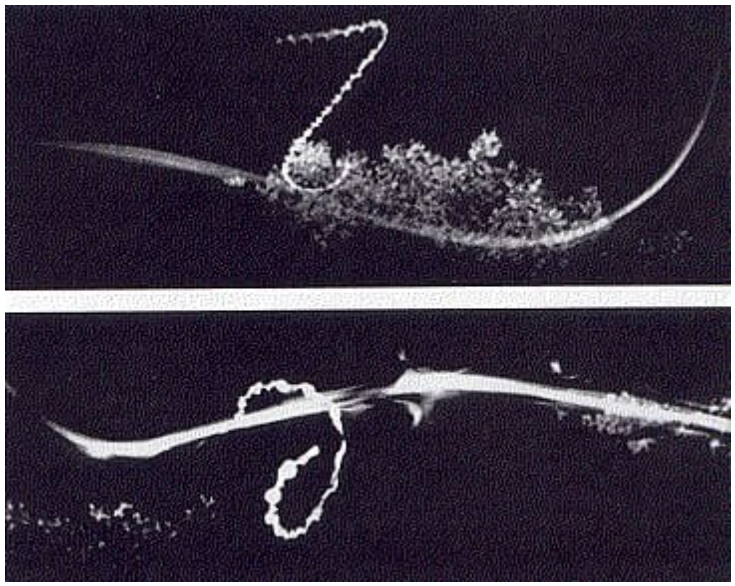


Figure 15.9.2.5 Hunter and hunted

Hunter and hunted. In the top photo, a moth (bright streak) takes successful evasive action upon detecting the approach of a bat (broad streak across the photo). (The diffuse image is a tree in the background.) In the bottom photo, the two streaks intersect, indicating that this time the moth was unable to escape capture by the bat. (Photos by Frederic A. Webster, courtesy of the late Prof. Kenneth D. Roeder.)

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15.9C: Vision

The human eye is wrapped in three layers of tissue:

- the **sclerotic coat.** This tough layer creates the "white" of the eye except in the front where it forms the transparent **cornea**. The corneaThe surface of the cornea is kept moist and dust-free by secretions from the tear glands.
  - admits light to the interior of the eye and
  - bends the light rays to that they can be brought to a focus.
- the **choroid coat.** This middle layer is deeply pigmented with **melanin**. It reduces reflection of stray light within the eye. The choroid coat forms the **iris** in the front of the eye. This, too, is pigmented and is responsible for eye "color". The size of its opening, the **pupil**, is variable and under the control of the autonomic nervous system. In dim light (or when danger threatens), the pupil opens wider letting more light into the eye. In bright light the pupil closes down. This not only reduces the amount of light entering the eye but also improves its image-forming ability (as does "stopping down" the iris diaphragm of a camera).
- the **retina** The retina is the inner layer of the eye. It contains the light receptors, the **rods** and **cones** (and thus serves as the "film" of the eye). The retina also has many interneurons that process the signals arising in the rods and cones before passing them back to the brain. **(Note:** the rods and cones are **not at the surface** of the retina but lie underneath the layer of interneurons.)

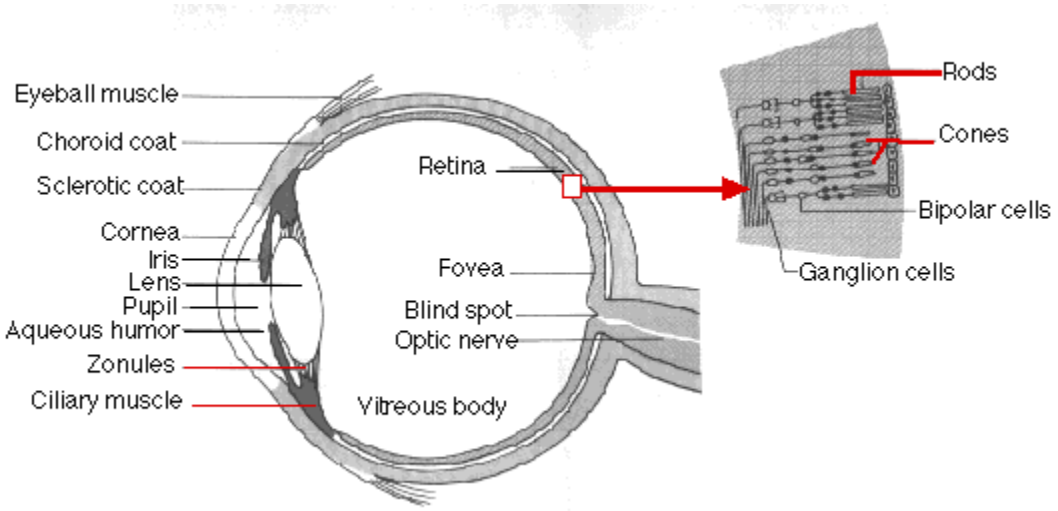


Figure 15.9.3.1 Human eye

The blind spot

All the nerve impulses generated in the retina travel back to the brain by way of the axons in the optic nerve (above). At the point on the retina where the approximately 1 million axons converge on the optic nerve, there are no rods or cones. This spot, called the blind spot, is thus insensitive to light.

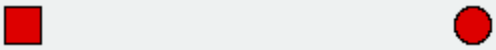


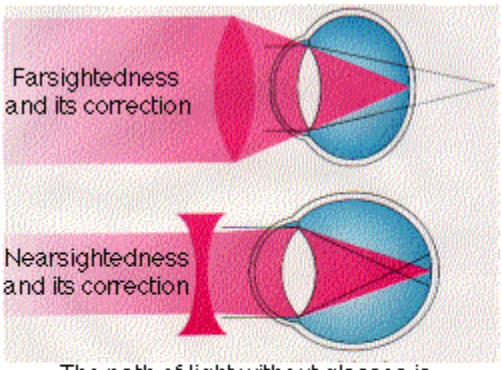
Figure 15.9.3.2 Blind spot

You can demonstrate the presence of the blind spot. Cover your right eye with your hand and stare at the red circle as you move closer to the screen (the square will disappear). Or cover your left eye and stare at the red square as you move.

The Lens

The lens is located just behind the iris. It is held in position by **zonules** extending from an encircling ring of muscle. When

this **ciliary muscle** is **relaxed**, its diameter increases, the zonules are put under tension, and the lens is flattened and when **contracted**, its diameter is reduced, the zonules relax, and the lens becomes more spherical. These changes enable the eye to adjust its focus between far objects and near objects.



The path of light without glasses is shown with black lines; with glasses, in pink. The lenses of modern eyeglasses are not so simple in shape as those shown here but function in the same way.

Figure 15.9.3.3 Eye corrections

Farsightedness. If the eyeball is too short or the lens too flat or inflexible, the light rays entering the eye — particularly those from nearby objects — will not be brought to a focus by the time they strike the retina. Eyeglasses with convex lenses can correct the problem. Farsightedness is called **hypermetropia**.

Nearsightedness. If the eyeball is too long or the lens too spherical, the image of distant objects is brought to a focus in front of the retina and is out of focus again before the light strikes the retina. Nearby objects can be seen more easily. Eyeglasses with concave lenses correct this problem by diverging the light rays before they enter the eye. Nearsightedness is called **myopia**.

Cataracts One or both lenses often become cloudy as one ages. When a cataract seriously interferes with seeing, the cloudy lens is easily removed and a plastic one substituted. The entire process can be done in a few minutes as an outpatient under local anesthesia.

The iris and lens divide the eye into two main chambers:

- the front chamber is filled with a watery liquid, the **aqueous humor**
- the rear chamber is filled with a jellylike material, the **vitreous body**

The Retina

Four kinds of light-sensitive receptors are found in the retina:

- **rods**
- three kinds of **cones**, each "tuned" to respond best to light from a portion of the spectrum of visible light
  - cones that absorb long-wavelength light (red)
  - cones that absorb middle-wavelength light (green)
  - cones that absorb short-wavelength light (blue)

This scanning electron micrograph (courtesy of Scott Mittman and David R. Copenhagen) shows rods and cones in the retina of the tiger salamander. Each type of receptor has its own special pigment for absorbing light. Each consists of a transmembrane protein called **opsin** coupled to the prosthetic group **retinal**. Retinal is a derivative of vitamin A (which explains why night blindness is one sign of vitamin A deficiency) and is used by all four types of receptors.

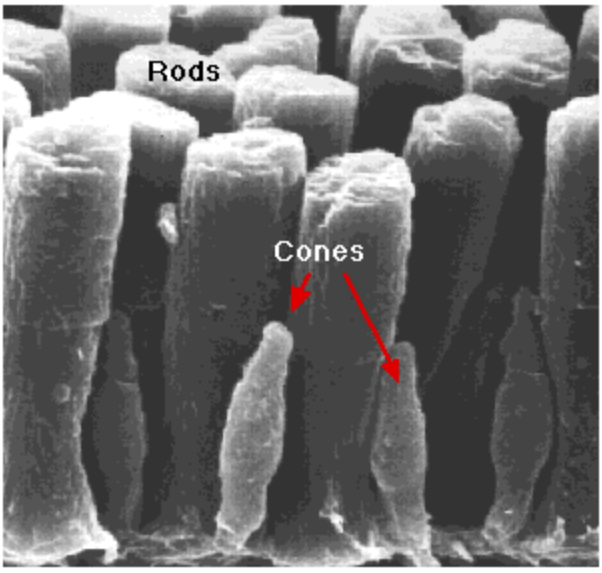


Figure 15.9.3.4 The retina

The amino acid sequence of each of the four types of opsin are similar, but the differences account for their differences in absorption spectrum. The retina also contains a complex array of interneurons:

- **bipolar cells** and **ganglion cells** that together form a path from the rods and cones to the brain
- a complex array of other interneurons that form synapses with the bipolar and ganglion cells and modify their activity.

Ganglion cells are always active. Even in the dark they generate trains of action potentials and conduct them back to the brain along the **optic nerve**. Vision is based on the modulation of these nerve impulses. There is not the direct relationship between visual stimulus and an action potential that is found in the senses of hearing, taste, and smell. In fact, action potentials are not even generated in the rods and cones.

Rod Vision

Rhodopsin is the light-absorbing pigment of the rods. This G-protein-coupled receptor (GPCR) is incorporated in the membranes of disks that are neatly stacked (some 1000 or more of them) in the outer portion of the rod. This arrangement is reminiscent of the organization of thylakoids, another light-absorbing device.



Fig.15.9.3.5 Rod cells of kangaroo bat

The electron micrograph (courtesy of Keith Porter) shows the rod cells of the kangaroo rat. The outer segments of the rods contain the orderly stacks of membranes which incorporate rhodopsin. The inner portion contain many mitochondria. The two parts of the rod are connected by a stalk (arrow) that has the same structure as a primary cilium. Although the disks are initially formed from the plasma membrane, they become separated from it. Thus signals generated in the disks must be transmitted by a chemical mediator (a "second messenger" called **cyclic GMP** (cGMP) to alter the potential of the plasma membrane of the rod. Rhodopsin consists of an opsin of 348 amino acids coupled to **retinal**. Like all G-protein-coupled receptors, opsin has 7 segments of alpha helix that pass back and forth through the lipid bilayer of the disk membrane.

Retinal

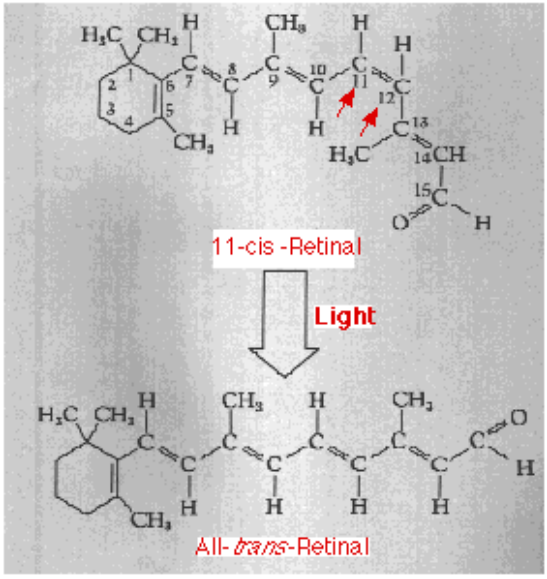
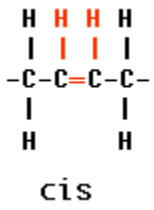
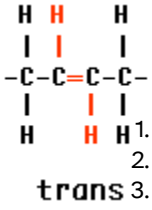


Figure 15.9.3.6 Retinal

Retinal consists of a system of alternating single and double bonds. In the dark, the hydrogen atoms attached to the #11 and #12 carbon atoms of retinal (red arrows) point in the same direction producing a kink in the molecule. This configuration is designated *cis*. When light is absorbed by retinal, the molecule straightens out forming the all-*trans* isomer.


cis


trans

This physical change in retinal triggers the following chain of events culminating in a change in the pattern of impulses sent back along the optic nerve.

1. Formation of all-*trans* retinal activates its **opsin**.
2. Activated rhodopsin, in turn, activates many molecules of a G protein called **transducin**.
3. Transducin activates an enzyme that breaks down **cyclic GMP**.
4. The drop in cGMP **closes** Na^+ channels in the plasma membrane of the rod. Because these positive ions can no longer enter, the interior of the cell becomes more negative (hyperpolarized) increasing its membrane potential from about –30 to some –70 mV.
5. This **slows** the release of the neurotransmitter glutamate at synapses between the rod and interneurons (e.g., bipolar cells).
6. This reduction in glutamate release activates some interneuron pathways, inhibits others.
7. The interplay of excited and inhibited interneurons modulates the spontaneous firing of the ganglion cells to which they are connected and gives rise to the ability of the retina to discriminate shapes.

So the retina is not simply a sheet of photocells, but a tiny brain center that carries out complex information processing before sending signals back along the optic nerve. In fact, the retina really **is** part of the brain and grows out from it during embryonic development.

Rod vision is acute but coarse

Rods do not provide a sharp image for several reasons.

- Adjacent rods are connected by gap junctions and so share their changes in membrane potential.
- Several nearby rods often share a single circuit to one ganglion cell.
- A single rod can send signals to several different ganglion cells.

So if only a single rod is stimulated, the brain has no way of determining exactly where on the retina it was. However, rods are extremely sensitive to light. A single photon (the minimum unit of light) absorbed by a small cluster of adjacent rods is sufficient to send a signal to the brain. So although rods provide us with a relatively grainy, colorless image, they permit us to detect light that is over a billion times dimmer than what we see on a bright sunny day.

Cone Vision

Although cones operate only in relatively bright light, they provide us with our sharpest images and enable us to see colors. Most of the 3 million cones in each retina are confined to a small region just opposite the lens called the **fovea**. So our sharpest and colorful images are limited to a small area of view. Because we can quickly direct our eyes to anything in view that interests us, we tend not to be aware of just how poor our peripheral vision is.

The three types of cones provide us the basis of color vision. Cones are "tuned" to different portions of the visible spectrum.

- red absorbing cones; those that absorb best at the relatively long wavelengths peaking at 565 nm
- green absorbing cones with a peak absorption at 535 nm
- blue absorbing cones with a peak absorption at 440 nm.

Retinal is the prosthetic group for each pigment. Differences in the amino acid sequence of their opsins accounts for the differences in absorption. The response of cones is not all-or-none. Light of a given wavelength (color), say 500 nm (green), stimulates all three types of cones, but the green-absorbing cones will be stimulated most strongly. Like rods, the absorption of light does not trigger action potentials but modulates the membrane potential of the cones.

Color Blindness

The term color blindness is something of a misnomer. Very few (~1 in 10^5) people cannot distinguish colors at all. Most "color-blind" people actually have abnormal color vision such as confusing the red and green of traffic lights. As high as 8% of the males in some populations have an inherited defect in their ability to discriminate reds and greens. These defects are found almost exclusively in males because the genes that encode the red-absorbing and green-absorbing opsins are on the X chromosome.

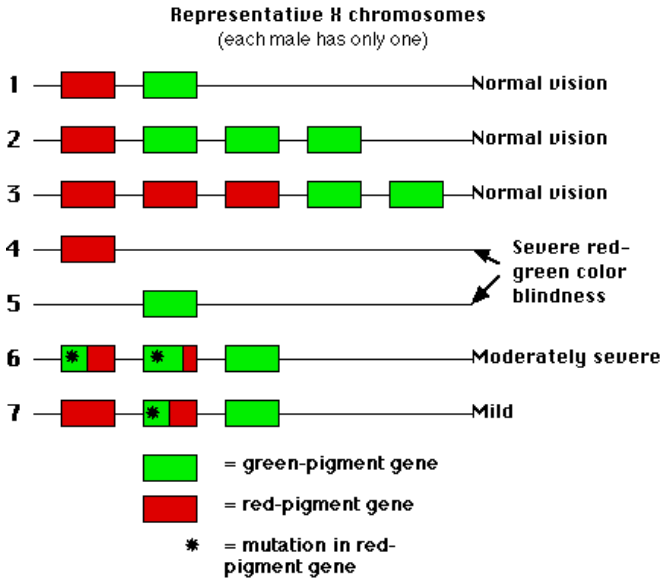


Figure 15.9.3.7 Red - Green genes

The X chromosome normally carries a cluster of from 2 to 9 opsin genes. The minimum basis for normal red-green vision is one gene whose opsin absorbs efficiently in the red and one that absorbs well in the green (chromosome **1** in the figure). Multiple copies of these genes are also fine (**2** and **3**). Males with either a "green gene" or "red gene" missing are severely color blind (**4** and **5**). However, if all the red genes carry mutations (this seldom seems to be the case for the green genes — nobody knows why), then they may have red-green color blindness that ranges from mild to severe depending on the particular mutations involved (**6**). The rule seems to be that the more the mutations shift the pigment towards green, the more serious the defect. However, a large number of mutations don't always produce serious defects. Multiple mutations in a single gene may offset each other producing only mild defects. And as long as one normal copy of each gene is present, the presence of additional mutated genes seldom produce a serious problem (**7**).

Why do some males have as many as 9 copies of genes encoding the red and green opsins, when two are enough? The sequences of the red and green genes are the same at 98% of their nucleotides. This high degree of similarity creates the risk of mismatches in synapsis during meiosis with unequal crossing over.

Blue vision

Abnormal blue sensitivity occasionally occurs in humans but is much rarer than abnormalities in red-green vision. The gene for the blue-cone opsin is located on chromosome 7. Thus this trait shows an autosomal pattern of inheritance being found in females as often as in males.

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15.9D: Processing Visual Information

This page discusses research by Stephen W. Kuffler and Nobel laureates David H. Hubel and Torsten N. Wiesel, focusing on how ganglion and LGN cells react to light. Ganglion cells vary their firing rates based on light location on the retina, while LGN cells are affected by cortical connections. In the visual cortex, cells respond to light edges, suggesting a convergence of inputs that filters information to recognize features. Hubel and Wiesel's work culminated in a Nobel Prize in 1981.

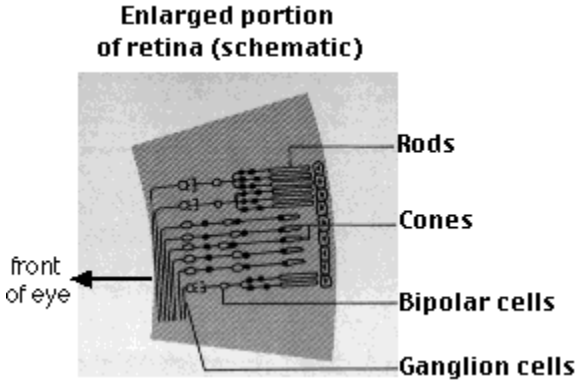


Figure 15.9.4.2 Ganglion cells

By inserting an electrode in a single ganglion cell, it was shown (by Stephen W. Kuffler) that

- Even in the dark, ganglion cells have a slow, steady rate of firing.
- Diffuse light directed on the retina has little effect on this rate.
- But a tiny **spot** of light falling on a small circular area of the retina can greatly increase the firing rate of some ganglion cells (left) while
- a spot directed around the perimeter of such an "**on**" area suppresses that ganglion cell (center).
- Light shining on both areas produces no effect (right).
- Other ganglion cells have a central "**off**" area surrounded by an "**on**" area.

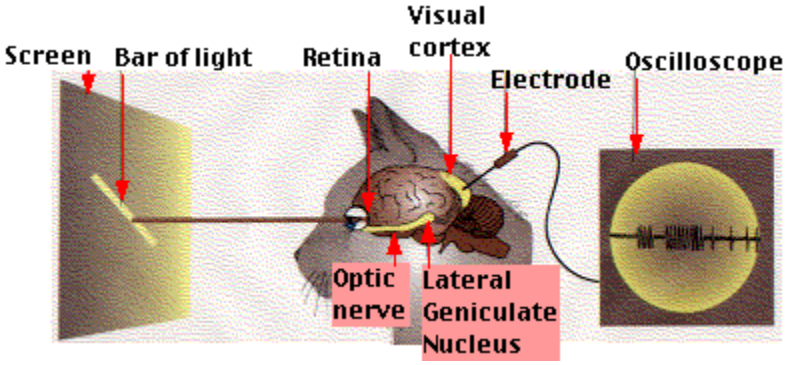


Figure 15.9.4.3 Lateral geniculate nucleus

Two associates of Kuffler, David H. Hubel and Torsten N. Wiesel inserted electrodes in these areas but instead of directing light into the eye, they projected images on a screen in front of the animal (an anesthetized cat or monkey). Using this procedure, they found that

- cells of the **lateral geniculate nucleus** (**LGN**) respond about the same way that ganglion cells do; that is, to **circular** spots of light.
- But the cells in the **visual cortex** receiving input from the LGN no longer respond to circles of light but only to **bars** of light (or dark) or to straight-line edges between dark and light areas.
- One of these "simple cortical cells" will only respond when the stimulus is directed at a particular area of the screen and at a specific angle. However, an ineffective position for one of these cortical cells is an effective position for another.

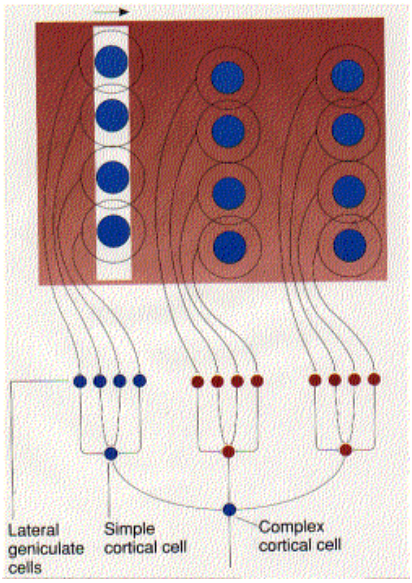


Figure 15.9.4.4 LGN

The diagram shows this mechanism by which the **circular response** areas of ganglion and **LGN** cells can be converted into the **rectangular response** areas found in the cells of the **visual cortex**. Other cells ("complex cortical cells") still want their edges oriented in one direction, but the edges can now be moved across the screen. As the figure shows, this can be explained if a set of simple cortical cells all responding to an edge of the same slope but each responsible for a different part of the visual field converge on a single "complex cortical cell". Thus these complex cortical cells continue to respond to the stimulus even though its absolute position on the retina changes.

While these studies provide only the tiniest glimpse into the workings of the brain, they provide some clues of what will be found:

- At each step of processing, the inputs of a number of interneurons are funneled into a single output.
- So, at each step, some of the information is selectively destroyed.
- A simple cortical cell, for example, fires only if a number of LGN cells converging on it are simultaneously active. Otherwise, the excitation dies out at the synapses.
- In this way, each level of the brain acts as a filtering device and, in doing so, provides a mechanism by which certain features of what might be a very complex stimulus can be discriminated..
- So instead of responding to particular impulses in particular circuits, the mammalian brain seems to respond to the spatial and temporal organization of many impulses passing along many converging circuits.

The importance of these studies was recognized by the award of a Nobel Prize in 1981 to Hubel and Wiesel (too late for Kuffler, who died in 1980).

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15.9E: Vision in Arthropods

The arthropod (e.g., insects, crustaceans) eye is built quite differently from the vertebrate eye (and mollusk eye). Arthropod eyes are called **compound eyes** because they are made up of repeating units, the **ommatidia**, each of which functions as a separate visual receptor. Each ommatidium consists of

- a **lens** (the front surface of which makes up a single **facet**)
- a transparent **crystalline cone**
- light-sensitive **visual cells** arranged in a radial pattern like the sections of an orange
- **pigment cells** which separate the ommatidium from its neighbors.

The pigment cells ensure that only light entering the ommatidium parallel (or almost so) to its long axis reaches the visual cells and triggers nerve impulses. Thus each ommatidium is pointed at just a single area in space and contributes information about only one small area in the field of view. There may be thousands of ommatidia in a compound eye with their facets spread over most of the surface of a hemisphere.

The photo, courtesy Carolina Biological Supply Company, shows the compound eye of ***Drosophila melanogaster***. The composite of all their responses is a **mosaic** image — a pattern of light and dark dots rather like the halftone illustrations in a newspaper or magazine. And just as in those media, the finer the pattern of dots, the better the quality of the image.

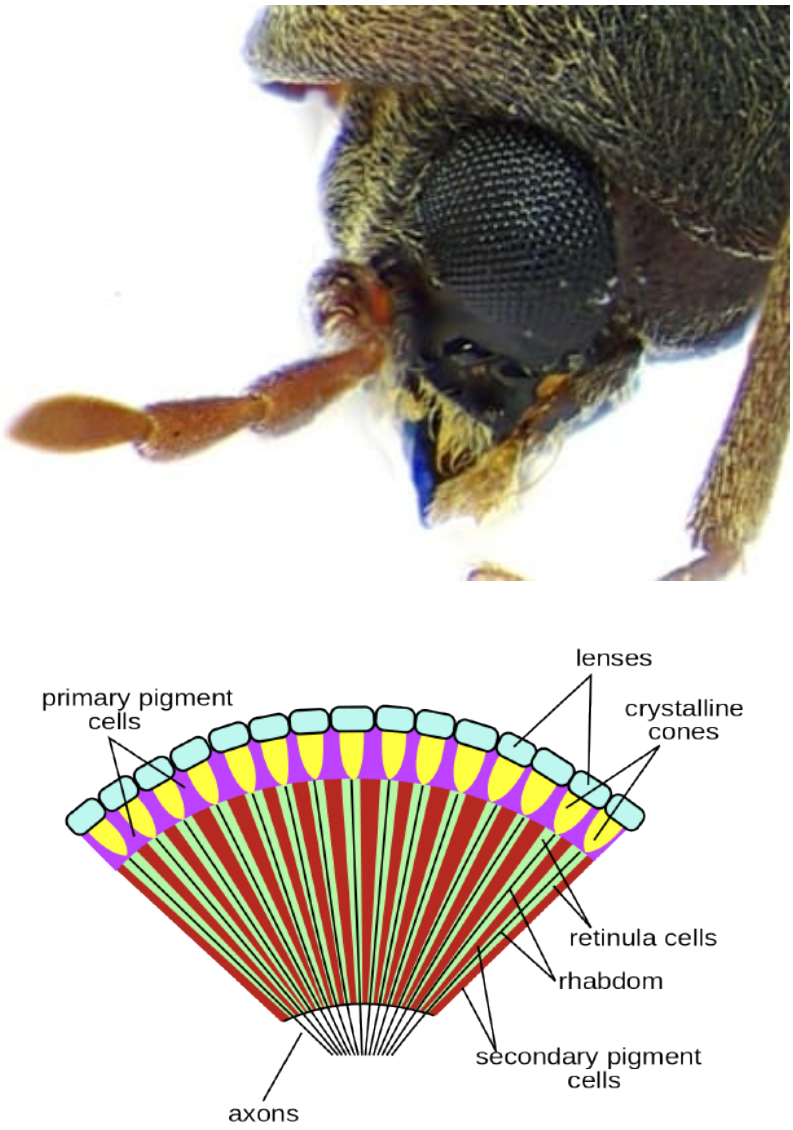


Figure $15.9E.1$: A photograph of the head of a woodworm beetle (Anobium punctatum) showing the compound eye and ommatidia. Photograph by Siga licensed under Creative Commons.

Grasshopper eyes, with relatively few ommatidia must produce a coarse, grainy image. The honeybee and dragonfly have many more ommatidia and a corresponding improvement in their ability to discriminate ("resolve") detail. Even so, the resolving ability of the honeybee eye is poor in comparison with that of most vertebrate eyes and only 1/60 as good as that of the human eye; that is, two objects that we could distinguish between at 60 feet (18 m) could only be discriminated by the bee at a distance of one foot (0.3 m).

Flicker effect

The compound eye is excellent at detecting motion. As an object moves across the visual field, ommatidia are progressively turned on and off. Because of the resulting "flicker effect", insects respond far better to moving objects than stationary ones. Honeybees, for example, will visit wind-blown flowers more readily than still ones.

Resolution and Sensitivity

Arthropods that are apt to be active in dim light (e.g., crayfish, praying mantis) concentrate the screening pigments of their ommatidia into the lower ends of the pigment cells. This shift enables light entering a single ommatidium at an angle to pass

into adjacent ommatidia and stimulate them also. With many ommatidia responding to a single area in the visual field, the image becomes coarser. The praying mantis probably can do little more than distinguish light and dark in the evening.

The shift in pigments does, however, make it more sensitive to light than it is in the daytime as more ommatidia can detect a given area of light.

Color vision

Some insects are able to distinguish colors. This requires two or more pigments, each of which absorbs best at a different wavelength. In the honeybee:

- four of the visual cells in each ommatidium respond best to yellow-green light (544 nm)
- two respond maximally to blue light (436 nm)
- the remaining two respond best to **ultraviolet light** (344 nm)

This system should enable the honeybee to distinguish colors (except red) and Figure $15.9E.2$ shows - behavioral studies verify this.

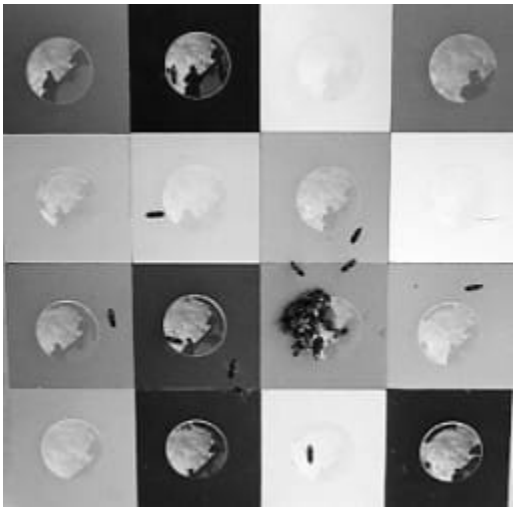


Figure $15.9E.2$: Color vision in bees Courtesy of Dr. M. Renner

The above picture shows a demonstration of color vision in honeybees. After a period of feeding from a dish placed on blue cardboard, the bees return to an empty dish on a clean blue card. They are able to distinguish the blue card from others of varying shades of gray.

Ultraviolet vision

Why ultraviolet vision? Television camera tubes are also sensitive to ultraviolet, as well as visible light, but their glass lens is opaque to ultraviolet. (This is why you cannot get tanned or synthesize *calciferol Vitamin D_2)* from the sunlight passing through window glass.) Using a special ultraviolet-transmitting lens (Figure $15.9E.3$), many insect-pollinated flowers appear to the honeybee quite different from the way they appear to us. The sharp contrasts between flowers that appear similar to us partly explains the efficiency with which honeybees secure nectar from only one species of flower at a time even when other species are also in bloom.



Figure $15.9E.3$: Images of a Mimulus flower in visible light (left) and ultraviolet light (right) showing a dark nectar guide that is visible to bees but not to humans. (CC BY-SA 3.0; Plantsurfer).

Monarch butterflies, which can migrate as much as 2500 miles (> 4000 km), navigate by ultraviolet light from the sun. When their view of the sun is through a filter that blocks out only its ultraviolet rays, their flight path becomes disoriented. Ultraviolet vision is not limited to animals with compound eyes. A few marsupials, rodents, a bat that feeds on nectar, and many birds have also been shown to have ultraviolet vision.

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15.9F: Heat, Cold, and Pain Receptors

This page explains how specific sensory neurons and their receptors detect heat, cold, and pain, detailing the roles of Aδ, C, and Aβ fibers in transmitting pain signals. It discusses various ion channels involved in temperature response and categorizes pain types—nociceptive, neuropathic, and visceral. Treatments like NSAIDs and opioids are examined, focusing on their mechanisms, effectiveness, and risks associated with opioid addiction and tolerance.

This page examines the detection of heat, cold, and pain. Why pain? Because at least some of the receptors of heat and cold, when the stimulus exceeds a certain threshold, transmit signals that the brain interprets as pain.

The Receptors

Few, if any, of the receptors of heat, cold, and pain are specialized transducers (in the way that, for example, the Pacinian corpuscle is). Rather they are sensory neurons whose plasma membrane contains transmembrane proteins that are ion channels that open in response to particular stimuli. A single neuron may contain several types of these ion channels and thus be able to respond to several types of stimuli. Like all sensory spinal neurons, their axons travel to a dorsal root ganglion of the spinal cord, where their cell bodies reside, and then on in to the gray matter of the spinal cord.

Three types of sensory neurons are found in the skin.

- Aδ ("A-delta") **fibers**
  - These are thinly-myelinated.
  - They transmit signals in response to heat and touch. If the stimulus exceeds a certain threshold, the brain interprets these as **acute pain**. This is "good pain" because it warns you to do something to take care of the problems, e.g., a hot saucepan.
- **C fibers**
  - These are unmyelinated and thus conduct impulses slowly.
  - C fibers also respond to heat and touch. If the stimulus exceeds a certain threshold, the brain interprets these as diffuse, dull, **chronic pain**. This is "bad pain" because it cannot be alleviated simply by removing the stimulus. It is pain generated by such things as damaged tissue or pain that remains after the stimulus that caused acute pain has been removed.
- **Aβ** ("A-beta") **fibers**
  - These are thickly-myelinated fibers.
  - They mostly respond to painless stimuli such as light **touch**.

Heat

There are several types of ion channels in the skin that respond to temperature. They are all transmembrane proteins in the plasma membrane that open to let **in** both calcium ions and sodium ions (the latter the source of the action potential). Between them, they cover a range of temperatures.

- TRPV4
  Warm (~27–34°C)
- **TRPV3**
  Warmer (~34–39°C)
- **TRPV1**
  Hot (≥43°C). Also activated by **capsaicin**, the active ingredient of hot chili peppers, by camphor, by acids (protons), and by pain-inducing products of inflammation.
- **TRPV2**
  Painfully hot (>52°C)

Knockout mice lacking the TRPV1 receptor not only do not avoid water with capsaicin in it but have a diminished response to heat and to substances that normal elicit itching. Birds also have TRPV1 receptors. Theirs also respond to heat (and acids), but do not respond to capsaicin. This must explain why birds happily eat hot chili peppers (and so disperse their seeds). The vampire bat, *Desmodus rotundus*, expresses normal TRPV1 receptors in the sensory neurons leading to the dorsal root ganglia,

and these respond normally to painful heat (> 43°C). However, these bats express a shortened version of TRPV1 (produced by alternative splicing) in their trigeminal nerves that run from the bat's upper lip and nose. The shortened receptors respond to a lower temperature (~30°C) enabling the bats to detect the warmth radiating from the skin of their victims.

Cold

Two candidate receptors:

- One, designated **TRPM8**, is a channel that admits Ca^{2+} and Na^{+} in response to moderate cold (<28°C) or **menthol** (the ingredient that gives mint its "cool" touch and taste). Knockout mice lacking the gene encoding the TRPM8 receptor do not avoid cold places as normal mice do.
- A second, designated **TRPA1**, responds to lower temperatures (<18°C). It also responds to several irritant chemicals eliciting signals that the brain interprets at pain. TRPA1 is found in the **hair cells** of the inner ear that respond to sound and changes in position.) However, TRPA1 knockout mice respond normally to cold and seem to have normal hearing so the precise role of these receptors is still uncertain for those stimuli.

  TRPA1 channels serve a different function in pit vipers like rattlesnakes. These cold-blooded animals detect warm-blooded prey using temperature-sensitive neurons at the base of pits in their head. The neurons contain TRPA1 channels that open wide when radiant heat entering the pit raises their temperature above 27°C.

Pain

When sensory nerve fibers are exposed to extremes, they signal pain. Pain receptors are also called **nociceptors**.

Processing Pathways

All the neurons in the skin are part of the sensory-somatic branch of the peripheral nervous system. Their axons pass into the dorsal root ganglion, where their cell body is located, and then on in to the gray matter of the spinal cord where they synapse with **interneurons**.

Several different neurotransmitters have been implicated in pain pathways. Three of them:

- glutamate. This seems to be the dominant neurotransmitter when the threshold to pain is first crossed. It is associated with acute ("good") pain.
- **substance P**. This peptide (containing 11 amino acids) is released by C fibers. It is associated with intense, persistent, chronic - thus "bad" pain.
- **glycine**. It **suppresses** the transmission of pain signals in the dorsal root ganglion. Prostaglandins potentiate the pain of inflammation by blocking its action.

Neuropathic Pain

This is pain caused by injury to the nerves themselves such as by mechanical damage, massive inflammation, and growing tumors.

Visceral Pain

The brain can also register pain from stimuli originating in sensory neurons of the autonomic nervous system. This so-called visceral pain is not felt in a discrete location as pain signals transmitted by the sensory-somatic system are.

Treating pain with drugs

The weapons presently available to reduce pain are many in number but few in types. They are

- Non-steroidal anti-inflammatory drugs (**NSAID**s)
- Opioids (also called opiates)

NSAIDs

Inflammation is caused by tissue damage and, among other things, causes pain. Damaged tissue releases **prostaglandins** and these are potent triggers of pain. Prostaglandins are 20-carbon organic acids synthesized from unsaturated fatty acids.

There are at least three key enzymes that synthesize prostaglandins:

- Cyclooxygenase 1 (**Cox-1**)
- Cyclooxygenase 2 (**Cox-2**)
- Cyclooxygenase 3 (**Cox-3**)

Most NSAIDs block the action of all three cyclooxygenases. They include:

- aspirin
- ibuprofen (Advil®, Motrin®)
- naproxen (Aleve®)
- and many others

Two NSAIDs celecoxib (Celebrex®) and rofecoxib (Vioxx®) were introduced in 1999 that selectively inhibit Cox-2 while leaving Cox-1 untouched. It was hoped that these would provide pain relief without the gastrointestinal side effects associated with the broad spectrum NSAIDs. However, the manufacturer of Vioxx® removed it from the market on 30 September 2004 because it increases the risk of heart attacks and strokes.

Acetaminophen (Tylenol®)

This is also a nonsteroidal anti-inflammatory drug but its mode of action is different from the others. It selectively inhibits Cox-3 and provides pain relief without irritating the stomach. It is particularly useful for

- people allergic to aspirin and its relatives
- avoiding the risk of Reye's syndrome that has been associated with giving aspirin to children with viral infections.

Opioids

Opioids are extremely effective pain killers but are also addictive so their use is surrounded by controversy and regulation.

Some examples:

- morphine
- codeine
- heroin
- methadone
- oxycodone

Opioids bind to receptors on interneurons in the pain pathways in the central nervous system. The natural ligands for these receptors are two **enkephalins** — each a pentapeptide (5 amino acids):

- Met-enkephalin (Tyr-Gly-Gly-Phe-Met)
- Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu)

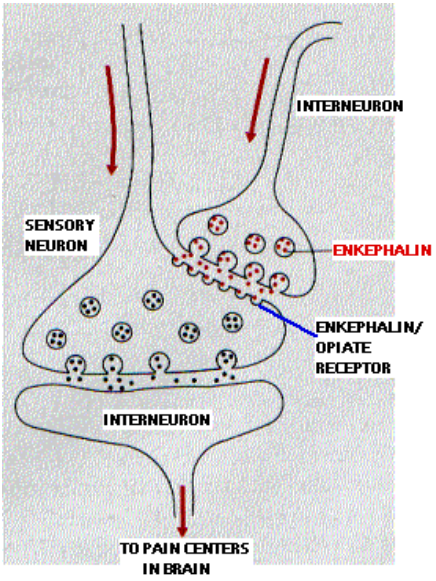


Figure 15.9.6.1 Pain synapse

The drawing shows how this mechanism might work. The activation of enkephalin synapses suppresses the release of the neurotransmitter (**substance P**) used by the sensory neurons involved in the perception of chronic and/or intense pain. The ability to perceive pain is vital. However, faced with massive, chronic, intractable pain, it makes sense to have a system that decreases its own sensitivity. Enkephalin synapses provide this intrinsic pain-suppressing system.

Morphine and the other opioids bind these same receptors. This makes them excellent pain killers.

However, they are also highly **addictive**.

- By binding to enkephalin receptors, they enhance the pain-killing effects of the enkephalins.
- A homeostatic reduction in the sensitivity of these synapses compensates for continued exposure to opioids.
- This produces **tolerance**, the need for higher doses to achieve the prior effect.
- If use of the drug ceases, the now relatively insensitive synapses respond less well to the soothing effects of the enkephalins, and the painful symptoms of **withdrawal** are produced.

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15.9G: Taste

This page explores the taste system, detailing five primary taste sensations—salty, sour, sweet, bitter, and umami. It explains that taste buds contain various cells responsive to these tastes, with specific receptors activating sensory neurons through distinct mechanisms: ion channels for salty, proton detection for sour, and G-protein-coupled receptors for sweet and bitter.

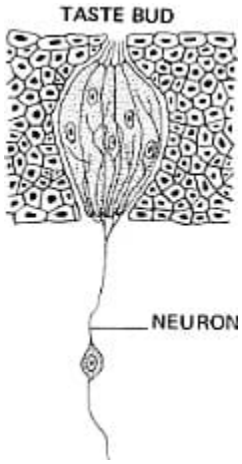


Figure 15.9.7.1 Taste bud

There are **five primary taste sensations**:

- salty
- sour
- sweet
- bitter
- umami

Properties of the taste system

- A single taste **bud** contains 50–100 **taste cells** representing **all 5** taste sensations (so the classic textbook pictures showing separate taste areas on the tongue are wrong).
- Each taste cell has **receptors** on its apical surface. These are transmembrane proteins which
  - admit the ions that give rise to the sensation of salty
  - bind to the molecules that give rise to the sensations of sweet, bitter, and umami
- A single taste cell seems to be restricted to expressing only a single type of receptor (except for bitter receptors).
- A stimulated taste receptor cell triggers action potentials in a nearby sensory neuron leading back to the brain.
- However, a single sensory neuron can be connected to several taste cells in each of several different taste buds.
- The sensation of taste like all sensations resides in the brain.
- And in mice, at least, the sensory neurons for four of the tastes (not sour) transmit their information to four discrete areas of the brain.

Salty

In mice, perhaps humans, the receptor for table salt (NaCl) is an ion channel that allows sodium ions (Na^+) to enter directly into the cell depolarizing it and triggering action potentials in a nearby sensory neuron.

In lab animals, and perhaps in humans, the hormone aldosterone increases the number of these salt receptors. This makes good biological sense:

- The main function of aldosterone is to maintain normal sodium levels in the body.
- An increased sensitivity to sodium in its food would help an animal suffering from sodium deficiency (often a problem for

ungulates, like cattle and deer).

Sour

Sour receptors detect the protons (H^+) liberated by sour substances (acids). This closes transmembrane K^+ channels which leads to depolarization of the cell, and the release of the neurotransmitter serotonin into its synapse with a sensory neuron.

Sweet

Sweet substances (like table sugar sucrose) bind to **G-protein-coupled receptors** (**GPCRs**) at the cell surface.

- Each receptor contains 2 subunits designated **T1R2** and **T1R3** and is coupled to **G proteins**.
- The complex of G proteins has been named **gustducin** because of its similarity in structure and action to the **transducin** that plays such an essential role in rod vision.
- Activation of gustducin triggers a cascade of intracellular reactions:
  - production of the second messengers inositol trisphosphate (IP3) and diacylglycerol (DAG)
  - this releases intracellular stores of Ca^{++}
  - this allows in the influx of Na^+ ions depolarizing the cell and causing the release of ATP
  - this triggers action potentials in a nearby sensory neuron.

The hormone leptin inhibits sweet cells by opening their K^+ channels. This hyperpolarizes the cell making the generation of action potentials more difficult. Could leptin, which is secreted by fat cells, be a signal to cut down on sweets?

Bitter

The binding of substances with a bitter taste, e.g., quinine, phenylthiocarbamide [PTC], also takes place on **G-protein-coupled receptors** that are coupled to gustducin and the signaling cascade is the same as for sweet (and umami).

Humans have genes encoding 25 different bitter receptors ("**T2R**s"), and each taste cell responsive to bitter expresses a number (4–11) of these genes. (This is in sharp contrast to the system in olfaction where a single odor-detecting cell expresses only a single type of odor receptor.)

Despite this — and still unexplained — a single taste cell seems to respond to certain bitter-tasting molecules in preference to others.

The sensation of taste — like all sensations — resides in the brain. Transgenic mice that

- express T2Rs in cells that normally express T1Rs (sweet) respond to bitter substances as though they were sweet
- express a receptor for a tasteless substance in cells that normally express T2Rs (bitter) are repelled by the tasteless compound

So it is the activation of hard-wired neurons that determines the sensation of taste, not the molecules nor the receptors themselves.

Umami

Umami is the response to salts of glutamic acid — like **monosodium glutamate** (**MSG**) a flavor enhancer used in many processed foods and in many Asian dishes. Processed meats and cheeses (proteins) also contain glutamate. The binding of amino acids, including glutamic acid, takes place on **G-protein-coupled receptors** that are coupled to heterodimers of the protein subunits **T1R1** and **T1R3**. The signaling cascade that follows is the same as it is for sweet and bitter.

Taste Receptors in Other Locations

Taste receptors have been found in several other places in the body. Examples:

- Bitter receptors (T2Rs) are found on the cilia and smooth muscle cells of the trachea and bronchi where they probably serve to expel inhaled irritants;
- Sweet receptors (T1Rs) are found in cells of the duodenum. When sugars reach the duodenum, the cells respond by releasing incretins. These cause the beta cells of the pancreas to increase the release of insulin.

So the function of "taste" receptors appears to be the detection of chemicals in the environment - a broader function than simply taste.

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15.9H: Olfaction - The Sense of Smell

This page explains that human smell involves sensory receptors in the olfactory epithelium, where odorant molecules interact with receptors on sensory neuron cilia. This interaction activates a signaling pathway that generates action potentials, allowing the brain to identify odors. Each olfactory neuron expresses one receptor gene, enabling the detection of up to a trillion distinct scents through combinatorial receptor activation patterns.

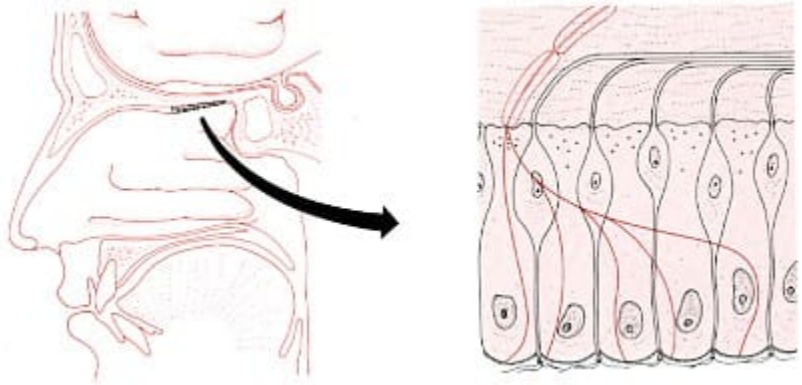


Figure 15.9.8.1 Olfactory epithelium

Smell depends on sensory receptors that respond to airborne chemicals. In humans, these **chemoreceptors** are located in the **olfactory epithelium** — a patch of tissue about the size of a postage stamp located high in the nasal cavity. The olfactory epithelium is made up of three kinds of cells:

- **sensory neurons** each with a **primary cilium**
- supporting cells between them
- **basal cells** that divide regularly producing a fresh crop of sensory neurons to replace those that die (and providing an exception to the usual rule that neurons seldom are replaced)

The sequence of events

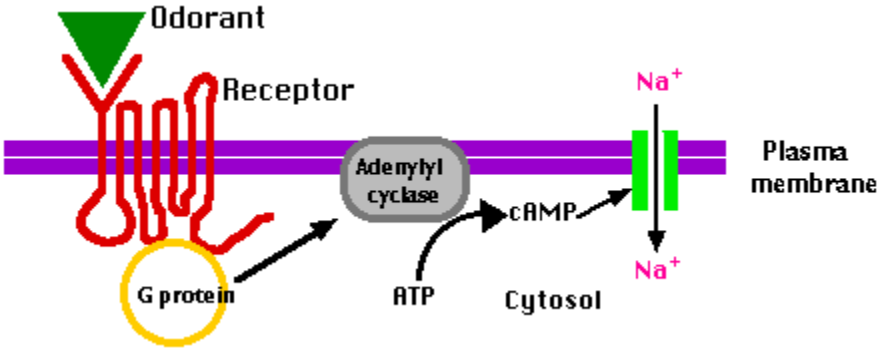


Figure 15.9.8.2 Olfaction

- The cilia of the sensory neurons are immersed in a layer of mucus. **Odorant** molecules (molecules that we can smell) dissolve in the mucus.
- These then **bind to receptors** on the cilia. These are "7-pass" transmembrane proteins.
- Binding of the odorant activates a **G protein** coupled to the receptor on its cytoplasmic side.
- This activates **adenylyl cyclase**, an enzyme embedded in the plasma membrane of the cilia.

- Adenylyl cyclase catalyzes the conversion of ATP to the "second messenger" cyclic AMP (**cAMP**) in the cytosol.

). Some other examples of GPCRs:

- receptors of peptide hormones
- taste receptors
- the light receptor rhodopsin
- $GABA_B$ receptors at certain synapses in the brain

- cAMP opens up ligand-gated **sodium channels** for the facilitated diffusion of Na^+ into the cell
- The influx of Na^+ reduces the potential across the plasma membrane.
- If this depolarization reaches threshold, it generates an action potential.
- The action potential is conducted back along the **olfactory nerve** to the brain.
- The brain evaluates this and other olfactory signals reaching it as a particular odor.

How can one kind of cell enable us to discriminate among so many different odors?

Humans can discriminate between hundreds, perhaps thousands, of different odorant molecules, each with its own structure. How can one kind of cell provide for this?

- The mechanism:

  Although a single olfactory neuron contains over a thousand receptor genes, there is only a single enhancer capable of binding to the promoters of these genes and turning them on. There are, of course, two alleles of the enhancer but only one is active (one is methylated; the other is not). Presumably, when the active enhancer encounters the promoter of an olfactory gene, it turns it on and ceases its search. Thus only one olfactory receptor gene gets to be expressed in a single cell, but which one is a matter of chance.

Now we have a mechanism for discriminating among a thousand or so odorants. However,

- **Each receptor is probably capable of binding to several different odorants** — some more tightly than others. (The cells described above also responded — although more weakly — to 3 related odorants.)
- **Each odorant is capable of binding to several different receptors.**

This provides the basis for **combinatorial diversity**. It would work like this:

Assume that

- **Odorant A** binds to receptors on **neurons #3, #427, and #886**.
- **Odorant B** binds to receptors on **neurons #2, #427, and #743**.

The brain then would interpret the two different patterns of impulses as separate odors.
This mechanism appears capable of discriminating between as many as a trillion different mixtures of odorants.

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15.9I: Electric Organs and Electroreceptors

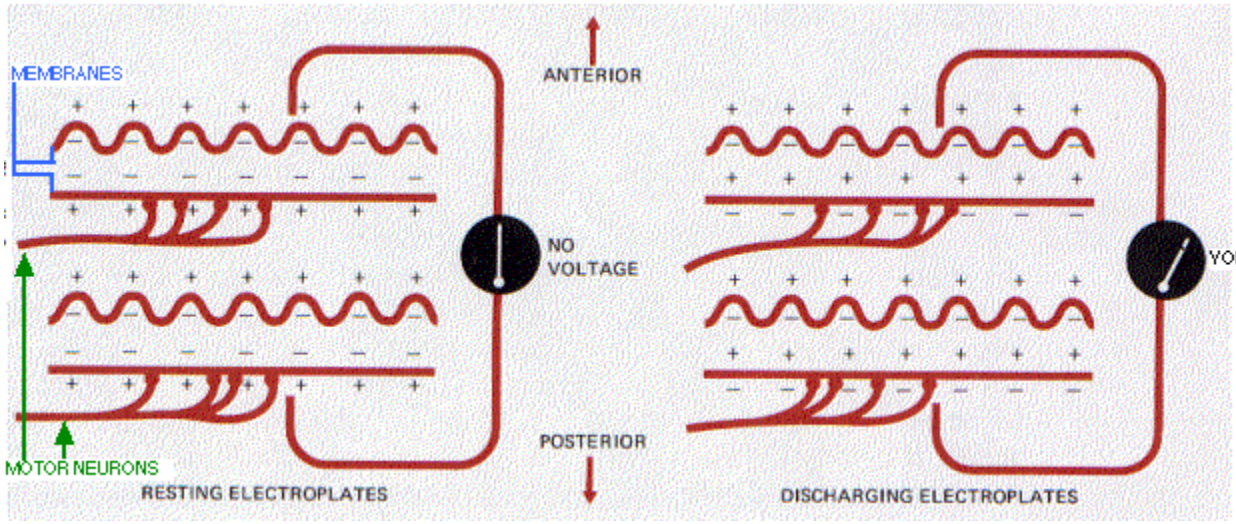


Figure 15.9.9.1 Electroplate

At rest, the interior of each electrocyte, like a nerve or muscle cell, is negatively charged with respect to the two exterior surfaces. The potential is about 0.08 volt, but because the charges alternate, no current flows. When a nerve impulse reaches the posterior surface, the inflow of sodium ions momentarily reverses the charge just as it does in the action potential of nerves and muscles. (In most fishes, electrocytes are, in fact, modified muscle cells.) Although the posterior surface is now negative, the anterior surface remains positive. The charges now reinforce each other and a current flows just as it does through an electric battery with the cells wired in "series".

With its several thousand electrocytes, the South American electric eel (*Electrophorus electricus*) produces voltages as high as 600 volts. The flow (amperage) of the current is sufficient (0.25–0.5 ampere) to stun, if not kill, a human. The pulse of current can be repeated several hundred times each second.

Powerful electric organs like those of the electric eel are used as weapons to stun prey as well as potential predators.

The Mechanism

In the 5 December 2014 issue of *Science*, Kenneth Catania describes his experiments that revealed how the electric eel captures its prey.

While exploring its environment, the eel emits a continuous series of **low**-voltage discharges. Periodically it interrupts these with a discharge of 2 or 3 **high**-voltage pulses. These cause nearby prey, e.g. a fish, to twitch. Within a tiny fraction of a second (20–40 ms) of detecting the twitch, the eel unleashes a volley (~400 per second) of high-voltage discharges that stun the prey enabling the eel to capture it.

Remarkably, both the twitch response and the immobilization are triggered by the prey's own motor neurons. A pair of pulses induces a brief contraction while a volley of discharges induces **tetanus**.

Although action potentials in the prey's motor neurons were not measured directly, two pieces of evidence support this mechanism.

1. The responses remained intact even when the brain and spinal cord of the prey were destroyed thus eliminating the possibility that the prey was relying on a sensory → cns → motor reflex.
2. Curare, which blocks the transmission of action potentials across the neuromuscular junction did block the prey's responses.

So hunting by the electric eel involves a preliminary 2 or 3 powerful pulses to - in Catania's words - answer the question "Are you

living prey?". If the answer is "yes", the prey is quickly stunned and ready to eat.

Weak Electric Organs

The electric organs of many fishes are too weak to be weapons. Instead they are used as signaling devices.

Many fishes, besides the electric eel, emit a continuous train of electric signals in order to detect objects in the water around them. The system operates something like an underwater radar and requires that the fishes also have electroreceptors (which are located in the skin). The presence of objects in the water distorts the electric fields created by the fish, and this alteration is detected by the electroreceptors.

Electric fishes use their system of transmitter and receiver for such functions as

- navigating in murky water and/or at night
- locating potential mates
- defense of their territory against rivals of the same species
- attracting other members of their species into schools

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15.9J: Magnetoreceptors

Evidence for an ability to alter their behavior in response to the earth's magnetic field has been found in many animals, including sea turtles, birds, fish (especially common in those that migrate), honeybees, mice as well as in some **bacteria**.

Some examples:

- Homing pigeons become disoriented when magnets are placed at the sides of their head. However, this disorientation occurs only on cloudy days, suggesting that their ability to navigate by magnetic cues is a backup system.
- Woodmice, taken from their home territory to a new location 40 meters away, normally orient toward home (left side of figure). But if, while they are being moved, they are exposed to a magnetic field that is just the reverse of the earth's magnetic field — and they are not allowed to see the surrounding terrain — they orient away from their home (right side of figure).

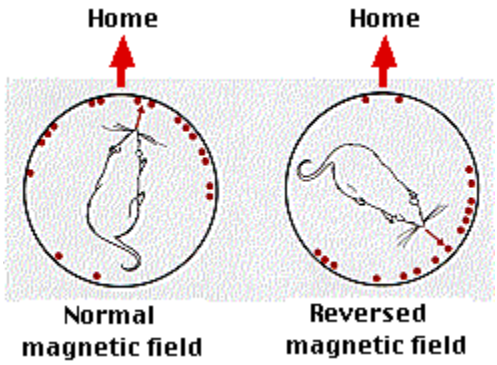


Figure 15.9.10.1 Magnetic sense

Left: orientation taken by individual woodmice after being removed from their home in a closed box. Right: same experiment except that the mice were subjected to a reversed magnetic field as they were moved from their home. Each dot represents the orientation taken by one mouse. The arrow within each circle indicates the average for all the mice. Mice transported in an open box so they can see landmarks orient correctly whether or not they are exposed to an abnormal magnetic field. (Based on the work of Mather and Baker, Nature, 291:152, 1981.)

Thrush nightingales (*Luscinia luscinia*) migrate in the fall from northern Europe to equatorial Africa. They interrupt their migration with a stopover in northern Egypt where they feed and gain weight. This stopover presumably provides them with the energy stores they need to fly without feeding across the Sahara Desert.

Fransson and colleagues report in the 1 November 2001 issue of **Nature** that when they confined naive birds (born in Sweden that spring) in Sweden but exposed them to a magnetic field characteristic of northern Egypt, the birds proceeded to put on weight as though they had arrived in Egypt (and three times more than control birds kept in the normal magnetic field of Sweden).

Receptors that detect magnetic fields

The location and mechanism of action of the receptors in these animals is still a puzzle. Microscopic grains of magnetite ($FeO{\cdot}Fe_2O_3$), a magnetic material, have been found in honeybees and pigeons, but whether and how these might function as receptors is not known.

Certain bacteria orient themselves in magnetic fields as weak as those of the earth and this is mediated by grains of magnetite within the cell. There is also evidence that birds and amphibians can supplement their magnetic sense using the interaction of light and magnetic fields on cryptochrome molecules in their **retina**. The ability of *Drosophila* to respond to magnetic fields depends on blue light and cryptochrome.

- In the absence of blue light, the flies do not respond to a magnetic field.
- Mutant flies that lack cryptochrome are likewise insensitive to magnetic fields.
- However, mutant flies whose own cryptochrome genes have been replaced by the human gene respond normally to a

magnetic field while exposed to blue light.

How humans detect magnetic fields

The jury is still out. There is some evidence that humans can detect the orientation of magnetic fields. Both cryptochrome and magnetite are found in humans, but their presence may have nothing to do with magnetoreception.

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15.10: MUSCLES

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- Topic hierarchy

15.10A: Bones

15.10B: Muscles

15.10C: Testing the Sliding-Filament Hypothesis

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15.10A: Bones

This page discusses the vertebrate endoskeleton's roles in support, protection, mineral storage, and blood cell production. It highlights the continuous remodeling of the human skeleton influenced by osteoclast and osteoblast activities, with hormonal regulation from growth hormone, parathyroid hormone, and estrogens.

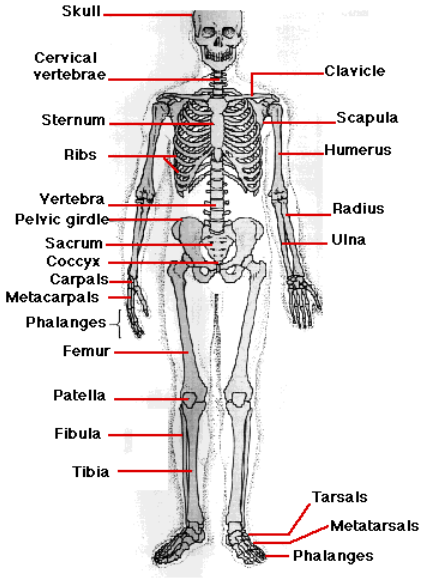


Figure 15.10.1.1 Human bone skeleton

The vertebrate body is supported by an **endoskeleton** made of cartilage and bone. (Sharks and their relatives use only cartilage.)

The bones of the human skeleton perform several functions:

- support
- protection of internal organs from mechanical damage (e.g., skull, ribs)
- reservoir of calcium and phosphate
- source of all the blood cells

Structure

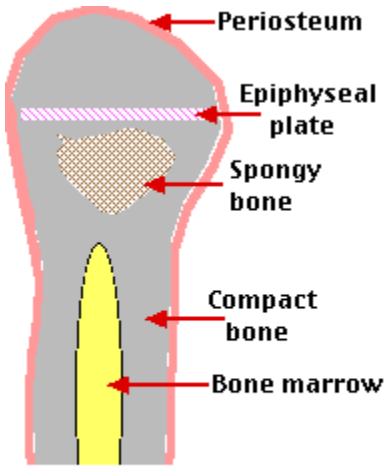


Figure 15.10.1.2 Bone structure

- Periosteum. A tissue covering the bone that brings blood and lymph vessels, as well as nerves, to it.
- **Compact bone** (also known as cortical bone). Dense deposits of minerals - mainly **calcium phosphate** and Type I collagen. These are arranged in concentric circles around a central **Haversian canal** through which blood and lymph vessels as well as nerves pass.

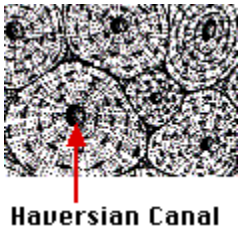


Figure 15.10.1.3 Haversian canal

- **Spongy bone** (also known as trabecular or cancellous bone). The mineral deposits are arranged as a system of struts. Bone marrow fill the spaces between.
- **Bone marrow.** Some bones, such as the femur, also contain a central cavity filled with bone marrow. Bone marrow contains the stem cells that gives rise to all the types of blood cells.
- **Epiphyseal plate.** Until the end of puberty, this disk of cartilage produces more cartilage which then is converted into more bone. In this way, the bone grows lengthwise.

Physiology

Looking at a skeleton, bone seems an inert thing. But in the living body it is anything but. The size and shape of bones not only changes during growth, but for the rest one's life it is continuously being remodeled in response to the stresses put on it. Approximately 10% of your bone mass is removed and replaced each year.

The remodeling of bone requires the coordinated activity of two types of cells:

- Osteoclasts are derived from stem cells in the bone marrow - the same ones produce monocytes and macrophages.

  Excess activity of osteoclasts (common after menopause in women) produces osteoporosis. The bones become weakened as cortical bone gets thinner and the spaces in spongy bone get larger.

  Reduced activity of osteoclasts produces **osteopetrosis**. This occurs when a person inherits a mutant version(s) of one or another of the genes needed for normal osteoclast function. Inhibition of osteoclast function causes too much bone to form leading to
  - extra-dense bone which actually is more brittle than normal bone thus leading to fractures
  - filling-in of the bone marrow cavity with bone thus interfering with the normal production of blood cells

Hormones and Bone

Many hormones affect bone growth and remodeling.

- Growth hormone (GH). As its name suggests, GH drives the growth of bones until the adult size is reached.
- Parathyroid hormone (PTH). It promotes the number and activity of **osteoblasts**.
- Estrogens. Until the end of puberty, estrogens are needed for maturation of the skeleton (in males as well as females). In women, after the menopause, taking supplemental estrogen slows up the bone loss that so often leads to osteoporosis. The estrogen induces FasL in **osteoclasts** causing them to self-destruct by apoptosis and in this way slows up the destruction of bone.
- Calcitonin and thyroid stimulating hormone (**TSH**), both of which inhibit the activity of osteoclasts.
- Calcitriol (1,25[OH]$_2$ vitamin D$_3$. Needed for the deposition of calcium into bone.
- Osteoprotegerin is a protein secreted by osteoblasts and their precursors (thus a cytokine) that also inhibits the production and activity of osteoclasts. Clinical trials of a recombinant version (made in E. coli) are underway as a possible treatment for various bone-weakening disorders like osteoporosis and multiple myeloma.
- Leptin, which regulates the balance between osteoblast and osteoclast activity.
- Serotonin. Secreted by the duodenum, serotonin suppresses osteoblasts (at least in mice). This may account for the bone-

weakening effect in humans of prolonged use of selective serotonin reuptake inhibitors (SSRIs).

Bone also produces hormones thus is itself an endocrine organ.

- Osteoblasts and their progeny produce a hormone — **fibroblast growth factor 23** (**FGF-23**) — which reduces the reabsorption of phosphate by the kidneys thus allowing more phosphate to pass out in the urine and lowering the phosphate level in the blood. FGF-23 is also called phosphatonin.
- Osteoblasts also secrete **osteocalcin**, a hormone that stimulates the beta cells of the pancreas to release insulin. This increases the uptake of glucose by skeletal muscles thus enhancing exercise capacity. Calcitonin also stimulates the Leydig cells of the testes to release testosterone. Osteocalcin crosses the blood-brain barrier and affects neurotransmitter levels, memory, and behavior in mice and may do so in humans.

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15.10B: Muscles

Animals use muscles to convert the chemical energy of ATP into mechanical work. Three different kinds of muscles are found in vertebrate animals.

- **Heart muscle** also called **cardiac muscle** makes up the wall of the heart. Throughout our life, it contracts some 70 times per minute pumping about 5 liters of blood each minute.
- **Smooth muscle** is found in the walls of all the hollow organs of the body (except the heart). Its contraction reduces the size of these structures. Thus it
  - regulates the flow of blood in the arteries
  - moves your breakfast along through your gastrointestinal tract
  - expels urine from your urinary bladder
  - sends babies out into the world from the uterus
  - regulates the flow of air through the lungs

  The contraction of smooth muscle is generally **not** under voluntary control.
- **Skeletal muscle**, as its name implies, is the muscle attached to the skeleton. It is also called **striated muscle**. The contraction of skeletal muscle is under voluntary control.

Anatomy of Skeletal Muscle

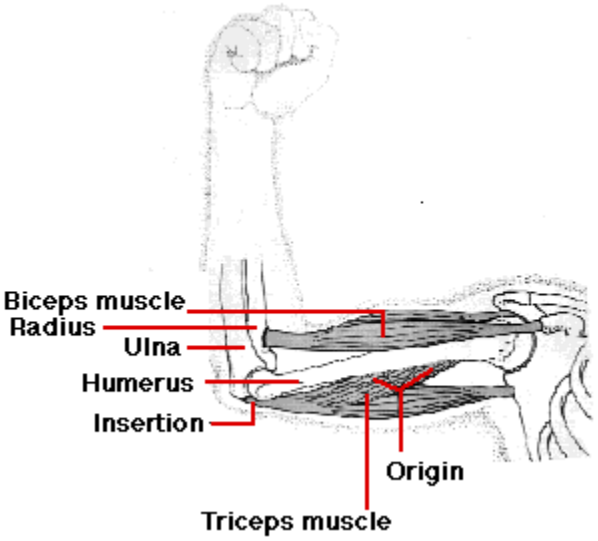


Figure 15.10.2.1 Forearm

A single skeletal muscle, such as the **triceps** muscle, is attached at its

- **origin** to a large area of bone; in this case, the **humerus**.
- at its other end, the **insertion**, it tapers into a glistening white **tendon** which, in this case, is attached to the **ulna**, one of the bones of the lower arm.

As the triceps contracts, the insertion is pulled toward the origin and the arm is straightened or extended at the elbow. Thus the triceps is an **extensor**. Because skeletal muscle exerts force only when it contracts, a second muscle - a **flexor** - is needed to flex or bend the joint. The **biceps** muscle is the flexor of the lower arm. Together, the biceps and triceps make up an **antagonistic pair** of muscles. Similar pairs, working antagonistically across other joints, provide for almost all the movement of the skeleton.

The Muscle Fiber

Skeletal muscle is made up of thousands of cylindrical muscle **fibers** often running all the way from origin to insertion. The fibers

are bound together by connective tissue through which run blood vessels and nerves.Each muscle fibers contains:

- an array of **myofibrils** that are stacked lengthwise and run the entire length of the fiber;
- **mitochondria**;
- an extensive **smooth endoplasmic reticulum (SER)**;
- **many nuclei** (thus each skeletal muscle fiber is a syncytium).

The multiple nuclei arise from the fact that each muscle fiber develops from the fusion of many cells (called **myoblasts**). The number of fibers is probably fixed early in life. This is regulated by **myostatin**, a cytokine that is synthesized in muscle cells (and circulates as a hormone later in life). Myostatin **suppresses** skeletal muscle development. (Cytokines secreted by a cell type that inhibit proliferation of that same type of cell are called **chalones**.) Cattle and mice with inactivating mutations in their myostatin genes develop much larger muscles. Some athletes and other remarkably strong people have been found to carry one mutant myostatin gene. These discoveries have already led to the growth of an illicit market in drugs supposedly able to suppress myostatin.

In adults, increased muscle mass comes about through an increase in the thickness of the individual fibers and increase in the amount of connective tissue. In the mouse, at least, fibers increase in size by attracting more myoblasts to fuse with them. The fibers attract more myoblasts by releasing the cytokine interleukin 4 (IL-4). Anything that lowers the level of myostatin also leads to an increase in fiber size.

Because a muscle fiber is not a single cell, its parts are often given special names such as

- **sarcolemma** for plasma membrane
- **sarcoplasmic reticulum** for endoplasmic reticulum
- **sarcosomes** for mitochondria
- **sarcoplasm** for cytoplasm

Although this tends to obscure the essential similarity in structure and function of these structures and those found in other cells.

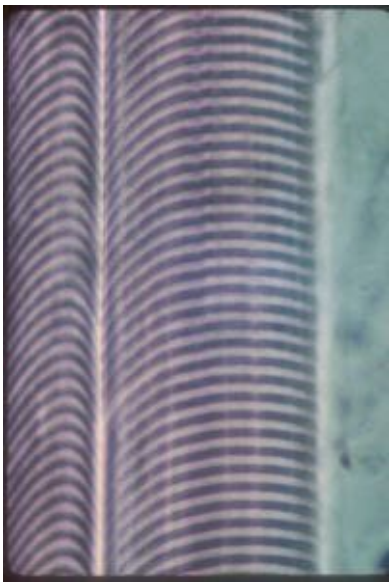


Figure 15.10.2.2 Striated muscle

The nuclei and mitochondria are located just beneath the plasma membrane. The endoplasmic reticulum extends between the myofibrils. Seen from the side under the microscope, skeletal muscle fibers show a pattern of cross banding, which gives rise to the other name: **striated muscle**. The striated appearance of the muscle fiber is created by a pattern of alternating dark **A bands** and light **I bands**.

- The A bands are bisected by the **H zone** running through the center of which is the **M line**.
- The I bands are bisected by the **Z disk**.

Each myofibril is made up of arrays of parallel **filaments**.

- The **thick filaments** have a diameter of about 15 nm. They are composed of the protein **myosin**.
- The **thin filaments** have a diameter of about 5 nm. They are composed chiefly of the protein **actin** along with smaller amounts of two other proteins - **troponin** and **tropomyosin**.

The anatomy of a sarcomere

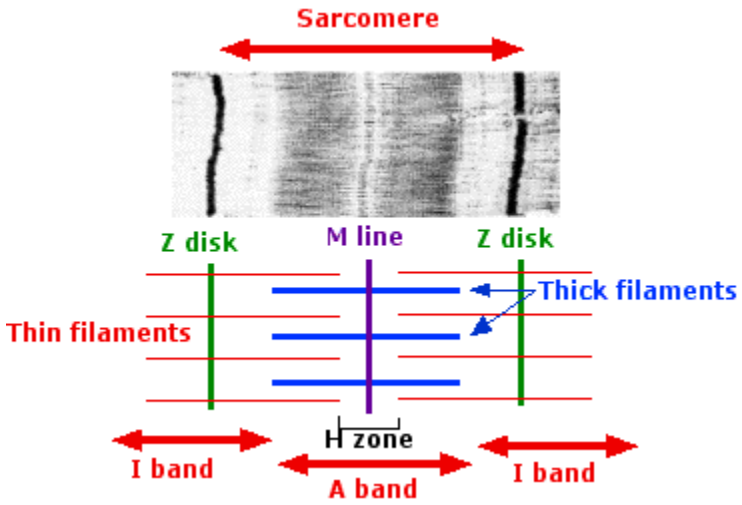


Figure 15.10.2.3 Sacromere

The entire array of thick and thin filaments between the Z disks is called a **sarcomere**.

- The **thick filaments** produce the dark **A band**.
- The **thin filaments** extend in each direction from the Z disk. Where they do not overlap the thick filaments, they create the light **I band**.
- The **H zone** is that portion of the A band where the thick and thin filaments do not overlap.
- The **M line** runs through the exact center of the sarcomere. Molecules of the giant protein, **titin**, extend from the M line to the Z disk. One of its functions is to provide elasticity to the muscle. It also provides a scaffold for the assembly of a precise number of myosin molecules in the thick filament (294 in one case). It may also dictate the number of actin molecules in the thin filaments.

Shortening of the sarcomeres in a myofibril produces the shortening of the myofibril and, in turn, of the muscle fiber of which it is a part. [This electron micrograph of a single sarcomere was kindly provided by Dr. H. E. Huxley.]

Activation of Skeletal Muscle

The contraction of skeletal muscle is controlled by the nervous system. The Dying Lioness (an Assyrian relief dating from about 650 B.C.) shows this vividly. Injury to the spinal cord has paralyzed the otherwise undamaged hind legs. In this respect, skeletal muscle differs from smooth and cardiac muscle. Both cardiac and smooth muscle can contract without being stimulated by the nervous system. Nerves of the autonomic branch of the nervous system lead to both smooth and cardiac muscle, but their effect is one of moderating the rate and/or strength of contraction.

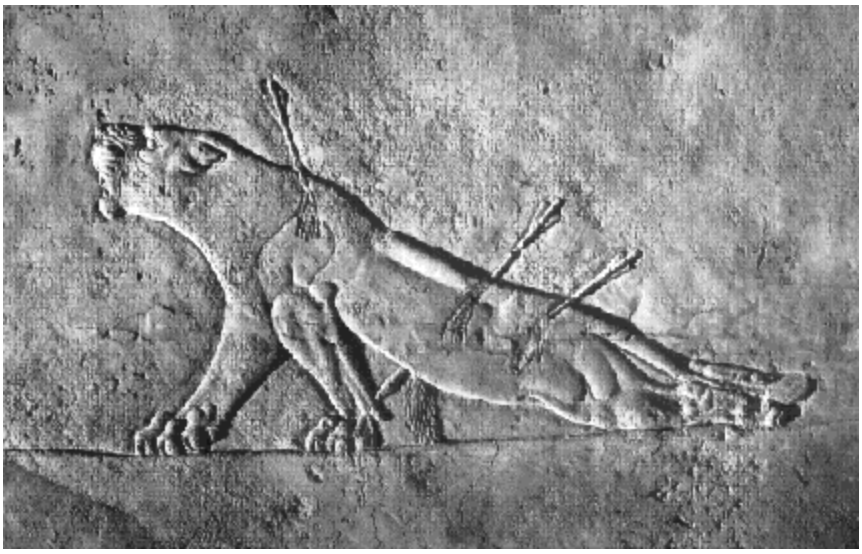


Figure 15.10.2.4: The Dying Lionesssupplied through the courtesy of The Trustees of the British Museum

The Neuromuscular Junction

Nerve impulses (action potentials) traveling down the motor neurons of the sensory-somatic branch of the nervous system cause the skeletal muscle fibers at which they terminate to contract. The junction between the terminal of a motor neuron and a muscle fiber is called the neuromuscular junction. It is simply one kind of synapse. (The neuromuscular junction is also called the myoneural junction.)

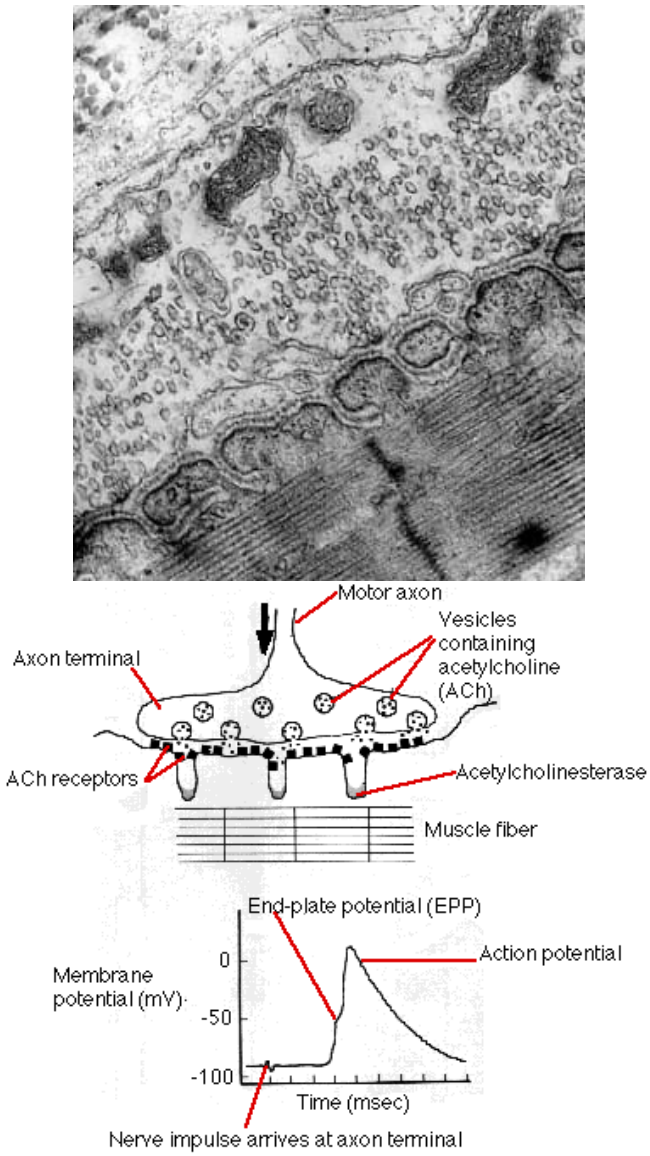


Figure 15.10.2.5 NM Junction

The terminals of motor axons contain thousands of vesicles filled with **acetylcholine** (**ACh**). Many of these can be seen in the electron micrograph on the left (courtesy of Prof. B. Katz).

When an action potential reaches the axon terminal, hundreds of these vesicles discharge their ACh onto a specialized area of postsynaptic membrane on the muscle fiber (the folded membrane running diagonally upward from the lower left). This area contains a cluster of transmembrane channels that are opened by ACh and let sodium ions (Na^+) diffuse in.

The interior of a resting muscle fiber has a **resting potential** of about –95 mV. The influx of sodium ions reduces the charge, creating an **end plate potential**. If the end plate potential reaches the **threshold** voltage (approximately –50 mV), sodium ions flow in with a rush and an **action potential** is created in the fiber. The action potential sweeps down the length of the fiber just as it does in an axon. No visible change occurs in the muscle fiber during (and immediately following) the action potential. This period, called the **latent period**, lasts from 3–10 msec.

Before the latent period is over,

- the enzyme **acetylcholinesterase**
  - breaks down the ACh in the neuromuscular junction (at a speed of 25,000 molecules per second)
  - the sodium channels close, and

    - the field is cleared for the arrival of another nerve impulse.
- the resting potential of the fiber is restored by an outflow of **potassium ions**.

The brief (1–2 msec) period needed to restore the resting potential is called the **refractory period**.

Tetanus

The process of contracting takes some 50 msec; relaxation of the fiber takes another 50–100 msec. Because the refractory period is so much shorter than the time needed for contraction and relaxation, the fiber can be maintained in the contracted state so long as it is stimulated frequently enough (e.g., 50 stimuli per second). Such sustained contraction is called **tetanus**.

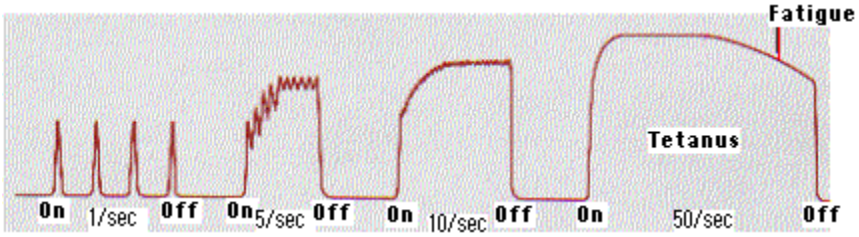


Figure 15.10.2.6 Tetanus

In the above figure:

- When shocks are given at 1/sec, the muscle responds with a single twitch.
- At 5/sec and 10/sec, the individual twitches begin to fuse together, a phenomenon called **clonus**.
- At 50 shocks per second, the muscle goes into the smooth, sustained contraction of tetanus.

Clonus and tetanus are possible because the refractory period is much briefer than the time needed to complete a cycle of contraction and relaxation. Note that the amount of contraction is greater in clonus and tetanus than in a single twitch.

As we normally use our muscles, the individual fibers go into tetanus for brief periods rather than simply undergoing single twitches.

The Sliding-Filament Model

Each molecule of myosin in the **thick filaments** contains a globular subunit called the **myosin head**. The myosin heads have binding sites for the **actin** molecules in the **thin filaments** and **ATP.** Activation of the muscle fiber causes the myosin heads to bind to actin. An allosteric change occurs which draws the thin filament a short distance (~10 nm) past the thick filament. Then the linkages break (for which ATP is needed) and reform farther along the thin filament to repeat the process. As a result, the filaments are pulled past each other in a ratchetlike action. There is no shortening, thickening, or folding of the individual filaments.

Electron microscopy supports this model.

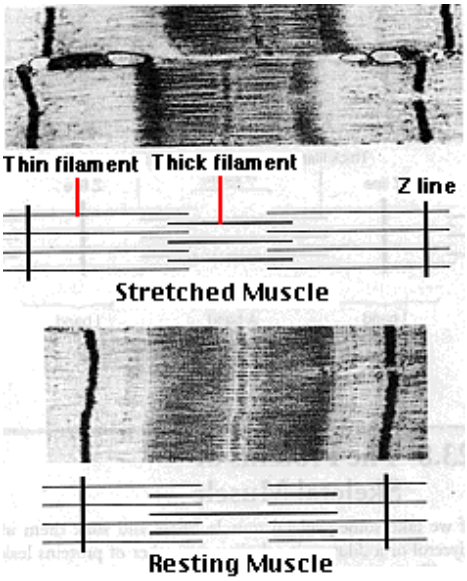


Figure 15.10.2.7 Sliding - Filament model courtesy of Dr. H. E. Huxley

As a muscle contracts,

- the Z disks come closer together
- the width of the I bands decreases
- the width of the H zones decreases
- there is no change in the width of the A band

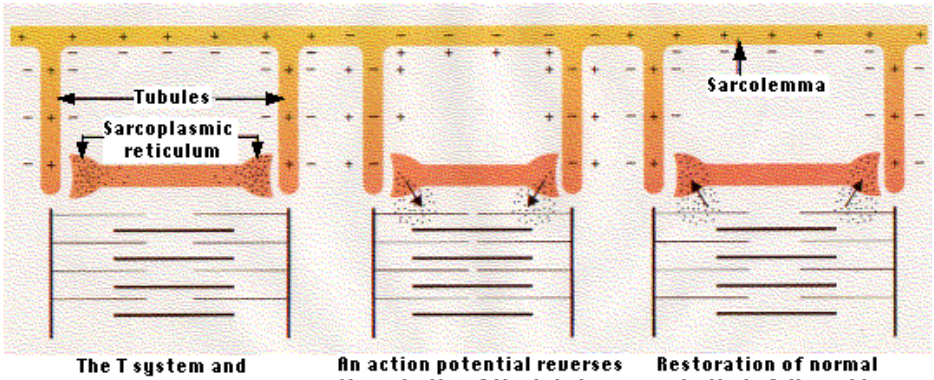
Conversely, as a muscle is stretched,

- the width of the I bands and H zones increases
- but there is still no change in the width of the A band

Coupling Excitation to Contraction

Calcium ions (Ca^{2+}) link action potentials in a muscle fiber to contraction.

- In resting muscle fibers, Ca^{2+} is stored in the endoplasmic (sarcoplasmic) reticulum.
- Spaced along the plasma membrane (**sarcolemma**) of the muscle fiber are inpocketings of the membrane that form **"T-tubules"**. These tubules plunge repeatedly into the interior of the fiber.
- The T-tubules terminate near the calcium-filled sacs of the **sarcoplasmic reticulum**.
- Each action potential created at the neuromuscular junction sweeps quickly along the sarcolemma and is carried into the T-tubules.



The T system and sarcoplasmic reticulum in resting muscle

An action potential reverses the polarity of the tubules releasing calcium ions (dots) from the sarcoplasmic reticulum. Their binding to troponin on the thin filaments "turns on" in interaction of actin and myosin, and the sarcomere shortens.

Restoration of normal polarity is followed by return of calcium to the sarcoplasmic reticulum and relaxation of the sarcomere.

Figure 15.10.2.8 Sacrolemma

- The arrival of the action potential at the ends of the T-tubules triggers the release of Ca^{2+}.
- The Ca^{2+} diffuses among the thick and thin filaments where it
- binds to **troponin** on the thin filaments.
- This turns on the interaction between actin and myosin and the sarcomere contracts.
- Because of the speed of the action potential (milliseconds), the action potential arrives virtually simultaneously at the ends of all the T-tubules, ensuring that all sarcomeres contract in unison.
- When the process is over, the calcium is pumped back into the sarcoplasmic reticulum using a **Ca^{2+} ATPase**.

Isotonic versus Isometric Contractions

If a stimulated muscle is held so that it cannot shorten, it simply exerts tension. This is called an **isometric** ("same length") contraction. If the muscle is allowed to shorten, the contraction is called **isotonic** ("same tension").

Motor Units

All motor neurons leading to skeletal muscles have branching axons, each of which terminates in a neuromuscular junction with a single muscle fiber. Nerve impulses passing down a single motor neuron will thus trigger contraction in all the muscle fibers at which the branches of that neuron terminate. This minimum unit of contraction is called the **motor unit**.

The size of the motor unit is small in muscles over which we have precise control. Examples:

- a single motor neuron triggers fewer than 10 fibers in the muscles controlling eye movements
- the motor units of the muscles controlling the larynx are as small as 2–3 fibers per motor neuron
- In contrast, a single motor unit for a muscle like the gastrocnemius (calf) muscle may include 1000–2000 fibers (scattered uniformly through the muscle).

Although the response of a motor unit is all-or-none, the strength of the response of the entire muscle is determined by the **number of motor units** activated. Even at rest, most of our skeletal muscles are in a state of partial contraction called **tonus**. Tonus is maintained by the activation of a few motor units at all times even in resting muscle. As one set of motor units relaxes, another set takes over.

Fueling Muscle Contraction

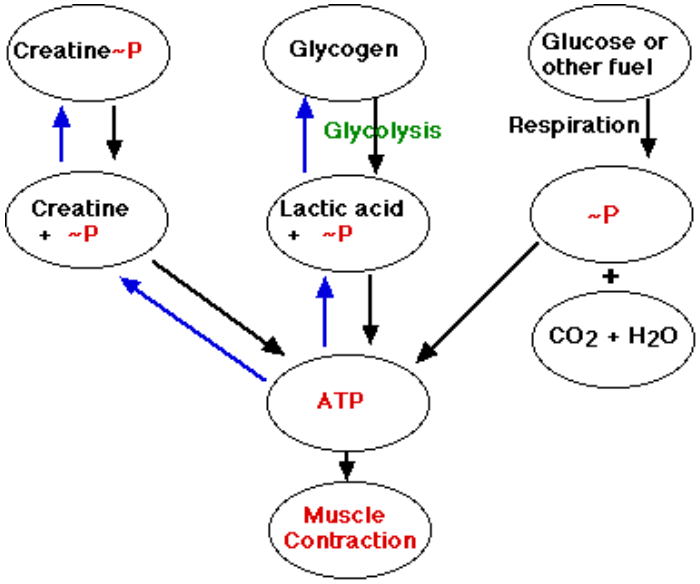


Figure 15.10.2.9 Creatine

ATP is the immediate source of energy for muscle contraction. Although a muscle fiber contains only enough ATP to power a few twitches, its ATP "pool" is replenished as needed. There are three sources of high-energy phosphate to keep the ATP pool filled.

- **creatine phosphate**
- **glycogen**
- **cellular respiration** in the mitochondria of the fibers.

Creatine phosphate

The phosphate group in creatine phosphate is attached by a "high-energy" bond like that in ATP. Creatine phosphate derives its high-energy phosphate from ATP and can donate it back to ADP to form ATP.

$$\text{Creatine phosphate} + \text{ADP} \leftrightarrow \text{creatine} + \text{ATP}$$

The pool of creatine phosphate in the fiber is about 10 times larger than that of ATP and thus serves as a modest reservoir of ATP.

Glycogen

Skeletal muscle fibers contain about 1% **glycogen**. The muscle fiber can degrade this glycogen by glycogenolysis producing glucose-1-phosphate. This enters the glycolytic pathway to yield two molecules of ATP for each pair of lactic acid molecules produced. Not much, but enough to keep the muscle functioning if it fails to receive sufficient oxygen to meet its ATP needs by respiration.

However, this source is limited and eventually the muscle must depend on cellular respiration.

Cellular respiration

Cellular respiration not only is required to meet the ATP needs of a muscle engaged in prolonged activity (thus causing more rapid and deeper breathing), but is also required afterwards to enable the body to resynthesize glycogen from the lactic acid produced earlier (deep breathing continues for a time after exercise is stopped). The body must repay its **oxygen debt**.

Type I vs. Type II Fibers

Several different types of muscle fiber can be found in most skeletal muscles: Type I and and 3 subtypes of Type II fibers. Each type differs in the myosin it uses and also in its structure and biochemistry.

Type I Fibers

- loaded with mitochondria
- depend on cellular respiration for ATP production
- fatty acids the major energy source
- resistant to fatigue
- rich in myoglobin and hence red in color (the "dark" meat of the turkey)
- activated by small-diameter, thus slow-conducting, motor neurons
- also known as "slow-twitch" fibers
- dominant in muscles used in activities requiring endurance (leg muscles) and those that depend on tonus, e.g., those responsible for posture

Type IIb Fibers

- few mitochondria
- rich in glycogen
- depend on creatine phosphate and glycolysis for ATP production
- fatigue easily with the production of lactic acid
- low in myoglobin hence whitish in color (the white meat of the turkey)
- activated by large-diameter, thus fast-conducting, motor neurons
- also known as "fast-twitch" fibers
- dominant in muscles used for rapid movement, e.g., those moving the eyeballs.

The other subtypes of Type II fibers have properties intermediate between those of Type IIb and Type I.

Most skeletal muscles contain some mixture of Type I and Type II fibers, but a single motor unit always contains one type or the other, never both.

In mice, the number of Type I vs Type II fibers can be changed with exercise and drug treatment. Whether the same holds true for humans remains to be seen. Perhaps training in humans does not alter the **number** of fibers of a particular type but may increase the diameter of one type (e.g., Type I in marathoners, Type IIb in weight lifters) at the expense of the other types.

Cardiac Muscle

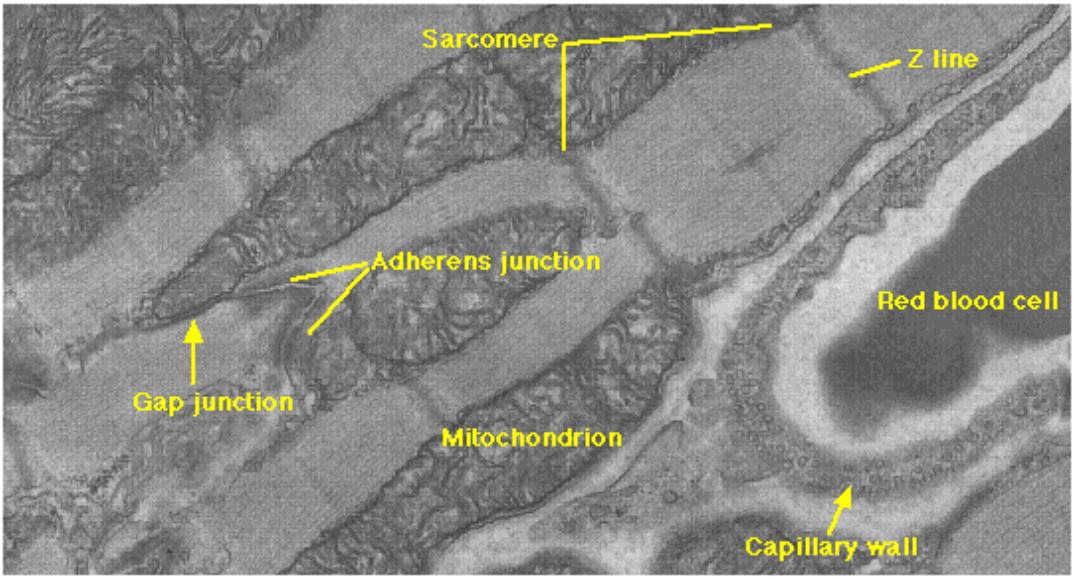


Figure 15.10.2.10 Cardiac muscle

Cardiac or heart muscle resembles skeletal muscle in some ways: it is striated and each cell contains sarcomeres with sliding filaments of actin and myosin. However, cardiac muscle has a number of unique features that reflect its function of pumping blood.

- The myofibrils of each cell (and cardiac muscle is made of single cells — each with a single nucleus) are branched.
- The branches interlock with those of adjacent fibers by adherens junctions. These strong junctions enable the heart to contract forcefully without ripping the fibers apart.

  This electron micrograph (reproduced with permission from Keith R. Porter and Mary A. Bonneville, *An Introduction to the Fine Structure of Cells and Tissues*, 4th ed., Lea & Febiger, Philadelphia, 1973) shows an adherens junction and several of the other features listed here.

- The action potential that triggers the heartbeat is generated within the heart itself. Motor nerves (of the autonomic nervous system) do run to the heart, but their effect is simply to modulate — increase or decrease — the intrinsic rate and the strength of the heartbeat. Even if the nerves are destroyed (as they are in a transplanted heart), the heart continues to beat.
- The action potential that drives contraction of the heart passes from fiber to fiber through gap junctions.
  - Significance: all the fibers contract in a synchronous wave that sweeps from the atria down through the ventricles and pumps blood out of the heart. Anything that interferes with this synchronous wave (such as damage to part of the heart muscle from a heart attack) may cause the fibers of the heart to beat at random — called **fibrillation**. Fibrillation is the ultimate cause of most deaths, and its reversal is the function of defibrillators that are part of the equipment in ambulances, hospital emergency rooms, and even on U.S. air lines.
- The **refractory period** in heart muscle is **longer** than the period it takes for the muscle to contract (systole) and relax (diastole). Thus **tetanus** is not possible (a good thing, too!).
- Cardiac muscle has a much richer supply of mitochondria than skeletal muscle. This reflects its greater dependence on cellular respiration for ATP.
- Cardiac muscle has little glycogen and gets little benefit from glycolysis when the supply of oxygen is limited.
  - Thus anything that interrupts the flow of oxygenated blood to the heart leads quickly to damage - even death - of the affected part. This is what happens in heart attacks.

Smooth Muscle

Smooth muscle is made of single, spindle-shaped cells. It gets its name because no striations are visible in them. Nonetheless, each smooth muscle cell contains **thick** (myosin) and **thin** (actin) filaments that slide against each other to produce contraction of the cell. The thick and thin filaments are anchored near the plasma membrane (with the help of intermediate filaments).

Smooth muscle (like cardiac muscle) does not depend on motor neurons to be stimulated. However, motor neurons (of the autonomic system) reach smooth muscle and can stimulate it or relax it depending on the neurotransmitter they release (e.g. noradrenaline or nitric oxide, NO).

Smooth muscle can also be made to contract

- by other substances released in the vicinity (paracrine stimulation)
  - Example: release of **histamine** causes contraction of the smooth muscle lining our air passages (triggering an attack of **asthma**)
- by hormones circulating in the blood
  - Example: oxytocin reaching the uterus stimulates it to contract to begin childbirth.

The contraction of smooth muscle tends to be slower than that of striated muscle. It also is often sustained for long periods. This, too, is called **tonus** but the mechanism is not like that in skeletal muscle.

Muscle Diseases

The Muscular Dystrophies (MD)

Together myosin, actin, tropomyosin, and troponin make up over three-quarters of the protein in muscle fibers. Some two dozen other proteins make up the rest. These serve such functions as attaching and organizing the filaments in the sarcomere and connecting the sarcomeres to the plasma membrane and the extracellular matrix. Mutations in the genes encoding these proteins may produce defective proteins and resulting defects in the muscles.

Among the most common of the muscular dystrophies are those caused by mutations in the gene for **dystrophin**. The gene for dystrophin is huge, containing 79 exons spread out over 2.4 million base pairs of DNA. Thus this single gene represents about 0.1% of the entire human genome (3×10^9 bp) and is almost half the size of the entire genome of E. coli!

- **Duchenne muscular dystrophy** (**DMD**)

  Deletions or nonsense mutations that cause a **frameshift** usually introduce premature termination codons (PTCs) in the resulting mRNA. Thus at best only a fragment of dystrophin is synthesized and DMD, a very severe form of the disease, results.

- **Becker muscular dystrophy** (**BMD**).

  If the deletion simply removes certain exons but preserves the correct reading frame, a slightly-shortened protein results that produces BMD, a milder form of the disease.

The gene for dystrophin is on the X chromosome, so these two diseases strike males in a typical X-linked pattern of inheritance.

A treatment for DMD

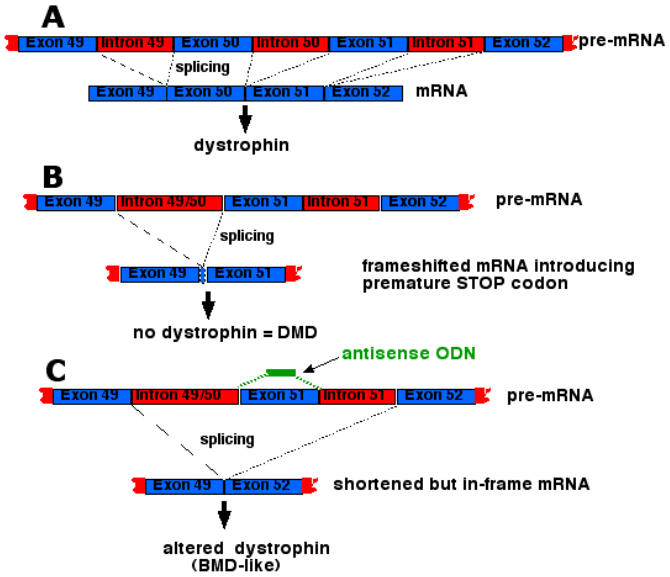


Figure 15.10.2.11 Exon skipping

Deletions of one or more exons in the huge dystrophin gene are the cause of most of the cases of DMD. Exon 50 is a particularly notorious offender. When it is deleted, splicing of the pre-mRNA introduces a frameshift which then introduces a premature termination codon resulting in no functional dystrophin synthesized ("**B**"). However, an antisense oligonucleotide targeted to exon 51 causes the splicing mechanism to skip over it resulting in the stitching together of exons 49 and 52. This restores the correct reading frame so that only a slightly-altered version of dystrophin is produced, i.e., a BMD-type dystrophin ("**C**"). Seventeen weeks of weekly injections of 12 young DMD patients in the Netherlands with the oligonucleotide caused their muscles to synthesize sufficient amounts of dystrophin to enable 8 of them to walk better than before. (See Goemans, N., et al., in the 21 April 2011 issue of **The New England Journal of Medicine**.

Three research groups have used the CRISPR-Cas9 genome editing system to remove a mutated exon in DMD mice. The treatment restored dystrophin synthesis and improved skeletal and cardiac muscle function in the mice.

Myasthenia Gravis

Myasthenia gravis is an autoimmune disorder affecting the neuromuscular junction. Patients have smaller end plate potentials (EPPs) than normal. With repeated stimulation, the EPPs become too small to trigger further action potentials and the fiber ceases to contract. Administration of an inhibitor of acetylcholinesterase temporarily can restore contractility by allowing more ACh to remain at the site.

Patients with myasthenia gravis have only 20% or so of the number of ACh receptors found in normal neuromuscular junctions. This loss appears to be caused by antibodies directed against the receptors. Some evidence:

- A disease resembling myasthenia gravis can be induced in experimental animals by immunizing them with purified ACh receptors.
- Anti-ACh receptor antibodies are found in the serum of human patients.
- Experimental animals injected with serum from human patients develop the signs of myasthenia gravis.
- Newborns of mothers with myasthenia gravis often show mild signs of the disease for a short time after their birth. This is the result of the transfer of the mother's antibodies across the placenta during gestation.

The reason some people develop autoimmune antibodies against the ACh receptor is unknown.

The Cardiac Myopathies

Cardiac muscle, like skeletal muscle, contains many proteins in addition to actin and myosin. Mutations in the genes for these may cause the wall of the heart to become weakened and, in due course, enlarged. Among the genes that have been implicated

in these diseases are those encoding:

- actin
- two types of myosin
- troponin
- tropomyosin
- myosin-binding protein C (which links myosin to titin)

The severity of the disease varies with the particular mutation causing it (over 100 have been identified so far) . Some mutations are sufficiently dangerous that they can lead to sudden catastrophic heart failure in seemingly healthy and active young adults.

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15.10C: Testing the Sliding-Filament Hypothesis

The sliding-filament model postulates that when skeletal (or cardiac) muscle contracts, the thin and thick filaments in each **sarcomere** slide along each other without their shortening, thickening, or folding and the strength of the relative motion between the thick and thin filaments is determined by the number of cross-bridges that can form between the two.

The Testing

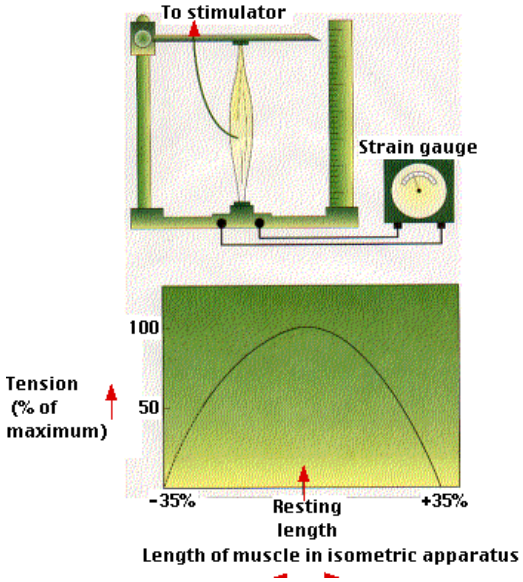


Figure 15.10.3.1 Isometric apparatus

This diagram shows apparatus with which to test the model.

- An isolated muscle (e.g., the calf muscle of a frog) placed in this apparatus cannot shorten when stimulated by an electric shock.
- Thus stimulation of the muscle produces an **isometric** ("same length") contraction.
- The muscle is placed in the apparatus and stretched to the desired length.
- It is then given a series of tetanizing shocks to measure its "active" tension; that is, the tension produced when it is stimulated.
- The strain gauge measures the tension exerted by the muscle.
- (Muscles are elastic, so if we stretch the muscle in putting it in the apparatus, it will already be exerting a "resting" tension.)
- Now let us plot the effect of muscle length on the active tension that is produced.

The Results

- The muscle produces the highest tension when held in the apparatus at the length it normally has in the intact animal.
- If held at longer (or shorter) lengths, the active tension produced is less.

You may conclude from this that nature knows best. And, in fact she does. If a muscle is surgically reattached to an animal so that its length is changed, the muscle gradually adapts to its new length and, after a few weeks, is able to exert its maximum isometric contractions at the new length.

The Interpretation

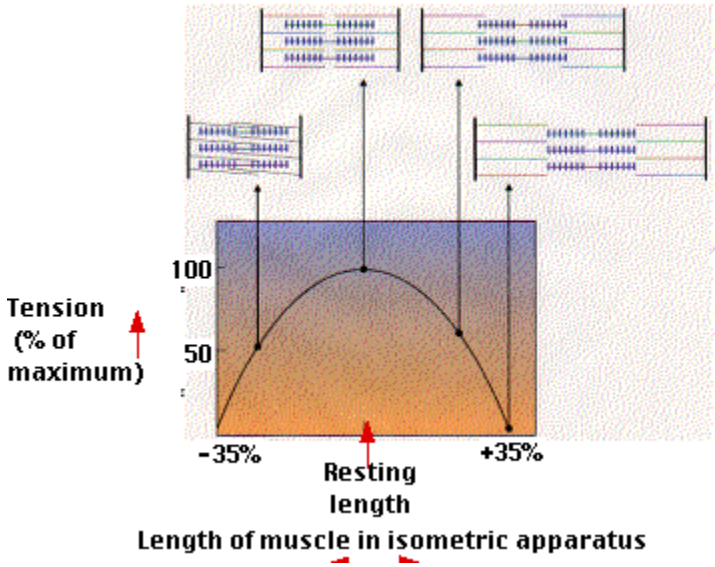


Figure 15.10.3.2 Interpretation of the results

- A muscle stretched beyond its normal length has less overlap between the thick and thin filaments.
- Thus fewer cross-bridges can form to slide the filaments against each other.
- In fact, if the muscle is stretched so far that the thin filaments are pulled entirely away from the thick filaments, the muscle exerts no tension at all.
- As for the effect of holding the muscle at shorter than normal length, the thin filaments extend so far across the sarcomere that they interact with cross-bridges exerting force the opposite way — reducing the tension generated.

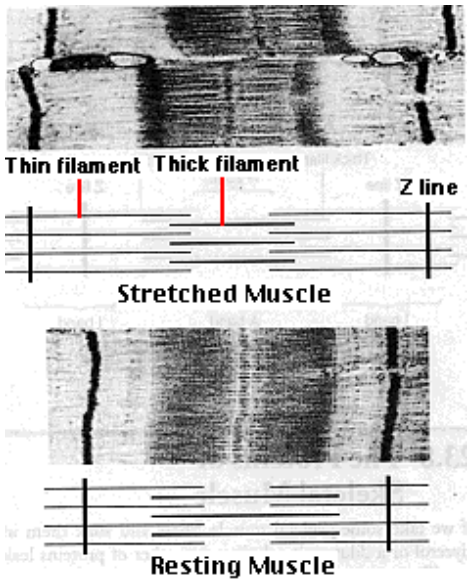


Figure 15.10.3.3 Striation patterns

These electron micrographs (courtesy of Dr. H. E. Huxley) show the pattern of striations in stretched muscle and resting muscle. In the stretched muscle, there is less overlap of the thick and thin filaments.

Consequently

- The light **I bands** and the **H zone** become wider.
- The width of the dark **A band** remains unchanged.
- We would not expect this pattern if sarcomere length involved a change in the length of the thick filaments.

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15.11: BEHAVIOR

- Topic hierarchy

15.11.1: Innate Behavior

15.11.2: Taxis

15.11.3: Learned Behavior

15.11.4: Long-Term Potentiation (LTP)

15.11.5: Honeybee Navigation

15.11.6: Avoiding Predation

15.11.7: Pheromones

15.11.8: Circadian Rhythms in Drosophila and Mammals

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15.11.1: Innate Behavior

Behavior is action that alters the relationship between an organism and its environment. Behavior may occur as a result of

- an external stimulus (e.g., sight of a predator)
- internal stimulus (e.g., hunger)
- or, more often, a mixture of the two (e.g., mating behavior)

It is often useful to distinguish between

- **innate behavior** = behavior determined by the "hard-wiring" of the nervous system. It is usually inflexible, a given stimulus triggering a given response. A salamander raised away from water until long after its siblings begin swimming successfully will swim every bit as well as they the very first time it is placed in the water. Clearly this rather elaborate response is "built in" in the species and not something that must be acquired by practice.
- **learned behavior** = behavior that is more or less permanently altered as a result of the experience of the individual organism (e.g., learning to play baseball well).
- However, careful analysis often reveals that any particular behavior is a combination of innate and learned components.

Examples of innate behavior:

- **taxes**
- **reflexes**
- **instincts**

Taxes

Some organisms respond to a stimulus by automatically moving directly toward or away from or at some defined angle to it. These responses are called taxes. They are similar to tropisms in plants except that actual locomotion of the entire organism is involved. Link to a detailed discussion.

Reflexes

The Withdrawal Reflex

When you touch a hot object, you quickly pull you hand away using the withdrawal reflex.

These are the steps:

- The stimulus is detected by **receptors** in the skin.
- These initiate nerve impulses in **sensory neurons** leading from the receptors to the spinal cord.
- The impulses travel into the spinal cord where the sensory nerve terminals synapse with **interneurons**.
    - Some of these synapse with **motor neurons** that travel out from the spinal cord entering mixed nerves that lead to the flexors that withdraw your hand.
    - Others synapse with inhibitory interneurons that suppress any motor output to extensors whose contraction would interfere with the withdrawal reflex.

The Stretch Reflex

The **stretch reflex** is examined (with a diagram) on a separate page. Link to it.

Instincts

Instincts are complex behavior patterns which, like reflexes, are inborn, rather inflexible, and valuable at adapting the animal to its environment. They differ from reflexes in their complexity. The entire body participates in instinctive behavior, and an elaborate series of actions may be involved.

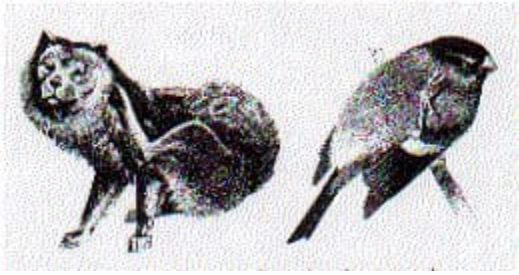


Figure 15.11.1.1 Instincts in animals and birds

The scratching behavior of a dog and a European bullfinch, shown here, is part of their genetic heritage. The widespread behavior of scratching with a hind limb crossed over a forelimb in common to most birds, reptiles, and mammals. (Picture courtesy of Rudolf Freund and Scientific American, 1958.) So instincts are inherited just as the structure of tissues and organs is. Another example.

- The African peach-faced lovebird carries nesting materials to the nesting site by tucking them in its feathers.
- Its close relative, the Fischer's lovebird, uses its beak to transport nesting materials.
- The two species can hybridize. When they do so, the offspring succeed only in carrying nesting material in their beaks. Nevertheless, they invariably go through the motions of trying to tuck the materials in their feathers first.

Foraging Behavior

Foraging for food is a crucial behavior for animals. Like all behavior, it requires the interaction of many components. Nonetheless, it turns out that in some animals, at least, foraging behavior can be altered by a single gene.

Drosophila melanogaster

The discovery of the genetic control of foraging in Drosophila began with the observations of Marla Sokolowski when she was an undergraduate biology student at the University of Toronto.

She noticed that Drosophila larvae, feeding in her culture vessels, displayed one of two distinct feeding patterns:

- "**rovers**" who moved rapidly over the surface of the culture medium
- "**sitters** who fed at a much more leisurely pace

She went on to find that this "bimodal" pattern of behavior

- continued when the larvae became adults
- was present in populations of wild fruit flies, not just in her laboratory colonies

After further years of research, she has shown that the behavior is under the control of a single gene, named *for* ("foraging"). Two alleles are present, at almost equal frequencies, that is, *for* is polymorphic.

- for^R, which is dominant
- for^S, the recessive
- About 70% of natural populations are rovers being either homozygous for for^R or heterozygous (for^R/for^S).
- Sitters are homozygous for for^S

Both alleles encode a **PKG**, a protein kinase (an enzyme that attaches phosphate groups to target proteins) that is activated by

the "second messenger" cyclic GMP (cGMP). The enzyme encoded by the *for*^{R} allele is more active than that encoded by *for*^{S}. She and her colleagues have succeeded in inserting *for*^{R} DNA into sitters who promptly become rovers.

Why this polymorphism? Why should alleles for two such different behaviors be maintained at such high frequency in the population?

One possible answer: it permits the population to thrive under varying food conditions:

- sitters are favored when food is abundant
- rovers are favored when competition for food is strong, such as in crowded cultures

Honeybees

Honeybees have their version of the *for* gene, called *Amfor* ("*Apis mellifera for*"). It, too, encodes a cGMP-dependent protein kinase (**PKG**). When worker bees first hatch, they remain in the hive tending to various housekeeping chores, such as feeding the larvae. But when they are 2–3 weeks old, they leave the hive and begin foraging for nectar and pollen. This change in behavior coincides with the increased expression of *Amfor*. When newly-hatched workers are treated with **cGMP**, the amount of PKG in their brains goes up and they quickly begin foraging instead of doing housekeeping.

Interaction of Internal and External Stimuli

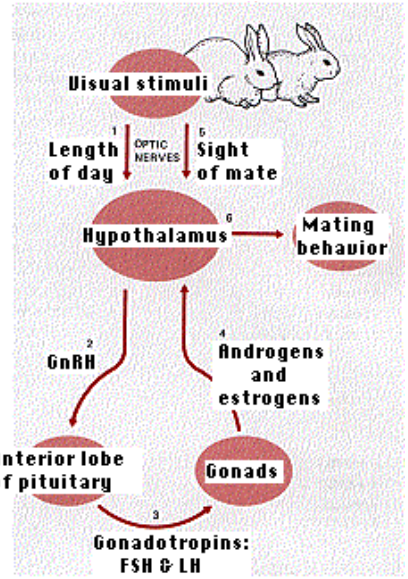


Figure 15.11.1.2 Mating rabbits

Instinctive behavior often depends on conditions in the internal environment. In many vertebrates courtship and mating behavior will not occur unless sex hormones (estrogens in females, androgens in males) are present in the blood. The target organ is a small region of the hypothalamus. When stimulated by sex hormones in its blood supply, the hypothalamus initiates the activities leading to mating. The level of sex hormones is, in turn, regulated by the activity of the anterior lobe of the pituitary gland.

The above figure outlines the interactions of external and internal stimuli that lead an animal, such as a rabbit, to see a sexual partner and mate with it.

Releasers of Instinctive Behavior

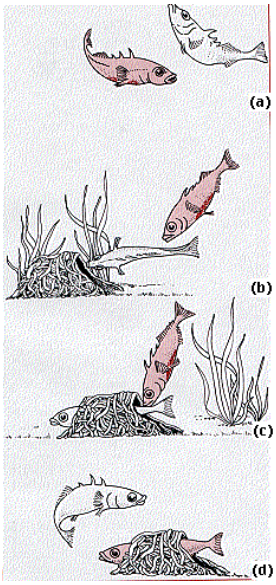


Figure 15.11.1.3 Breeding in a three-spined stickleback

Once the body is prepared for certain types of instinctive behavior, an external stimulus may be needed to initiate the response. N. Tinbergen (who shared the 1973 Nobel Prize with Konrad Lorenz and Karl von Frisch) showed that the stimulus need not necessarily be appropriate to be effective.

- During the breeding season, the female **three-spined stickleback** normally follows the red-bellied male (**a** in the figure) to the nest that he has prepared.
- He guides her into the nest (**b**) and then
- prods the base of her tail (**c**).
- She then lays eggs in the nest.
- After doing so, the male drives her from the nest, enters it himself, and fertilizes the eggs (**d**).
- Although this is the normal pattern, the female will follow almost any small red object to the nest, and once within the nest, neither the male nor any other red object need be present.
- Any object touching her near the base of her tail will cause her to release her eggs.

It is as though she were primed internally for each item of behavior and needed only one specific signal to release the behavior pattern. For this reason, signals that trigger instinctive acts are called **releasers**. Once a particular response is released, it usually runs to completion even though the stimulus has been removed. One or two prods at the base of her tail will release the entire sequence of muscular actions involved in liberating her eggs.

Chemical signals (e.g., pheromones) serve as important releasers for the social insects: ants, bees, and termites. Many of these animals emit several different pheromones which elicit, for example, alarm behavior, mating behavior, and foraging behavior in other members of their species.

The mammary glands of domestic rabbit mothers emit a pheromone that releases immediate nursing behavior by their babies (pups). A good thing, too, as mothers devote only 5–7 minutes a day to feeding their pups so they had better be quick about it.

The studies of Tinbergen and others have shown that animals can often be induced to respond to inappropriate releasers. For example, a male robin defending its territory will repeatedly attack a simple clump of red feathers instead of a stuffed robin that lacks the red breast of the males.

Although such behavior seems inappropriate to our eyes, it reveals a crucial feature of all animal behavior: animals **respond selectively** to certain aspects of the total sensory input they receive. Animals spend their lives bombarded by myriad sights, sounds, odors, etc. But their nervous system **filters** this mass of sensory data, and they respond only to those aspects that the evolutionary history of the species has proved to be significant for survival.

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15.11.2: Taxis

Some organisms respond to a stimulus by automatically moving directly toward or away from or at some defined angle to it. These responses are called taxes. They are similar to tropisms in plants except that actual locomotion of the entire organism is involved.

Chemotaxis

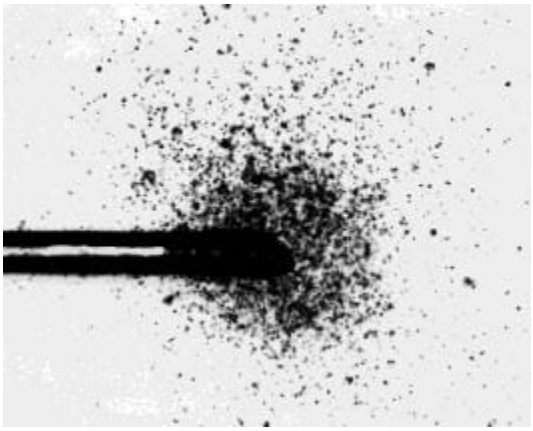


Figure 15.11.2.1: Chemotaxis. (courtesy of Dr. Julius Adler from Science, 26 December 1969)

When a capillary tube filled with glucose is placed in a medium containing ***E. coli***, the bacteria alter their locomotion so that they congregate near the opening of the tube. This chemotactic response does not depend on the bacteria being able to metabolize the substance although presumably that is the value under normal conditions. *E. coli* responds strongly to a number of organic molecules besides glucose, including galactose and the amino acids serine and aspartic acid. Figure Figure 15.11.2.1 shows how the bacteria have congregated at the opening of a capillary tube filled with a weak solution of an amino acid.

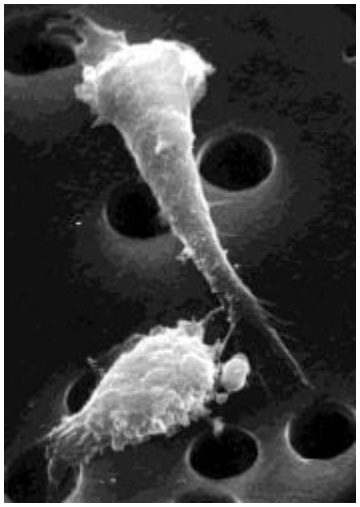


Figure 15.11.2.2: Chemokines. This scanning electron micrograph (courtesy of Drs. Ralph Snyderman and C. A. Daniels) shows two human monocytes migrating through the pores of a filter in response to a chemokine diffusing up from beneath.

Some of the motile cells of the immune system also display chemotaxis. The substances that attract them are called **chemokines** (for chemotactic cytokines).

Phototaxis

Photosynthetic microorganisms often display phototaxis.

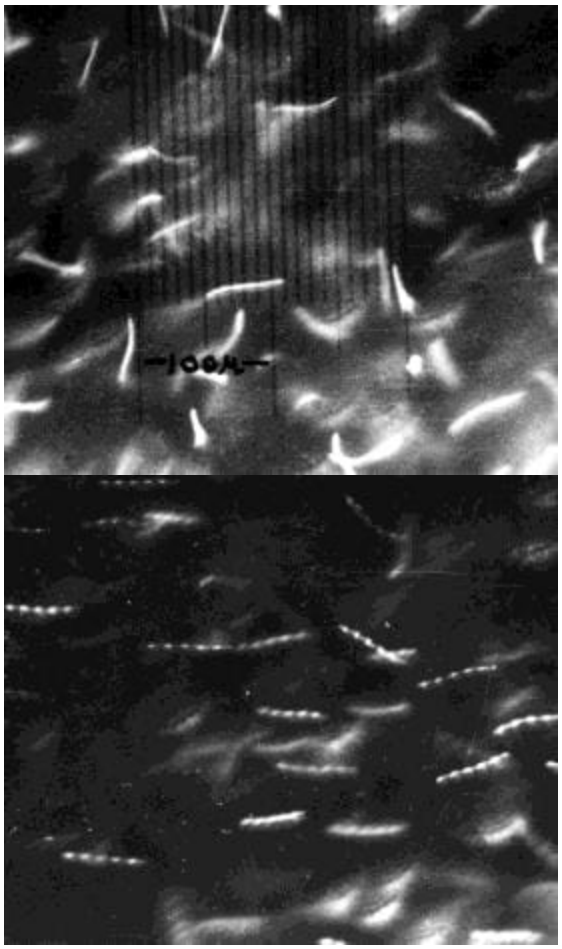


Figure 15.11.2.3: Phototaxis in green alga.These photographs (courtesy of Dr. Mary Ella Feinleib of Tufts University) shows phototaxis in the unicellular green alga Chlamydomonas.

In the left picture, randomly oriented tracks formed during 1/3 sec by the algae swimming about in red light to which the are insensitive. In the right picture, upon adding a beam of blue-green light from one side, the tracks become oriented in its direction.

Magnetotaxis

Several species of bacteria swim in the direction of magnetic lines of force. Because of the inclination of the earth's magnetic lines of force, this behavior causes the bacterium to swim downward and thus to return to the sediments in which it lives. For an organism as tiny as a bacterium, gravity is of no consequence. So here is an alternate mechanism by which dislodged bacteria can find their way back into their normal habitat.

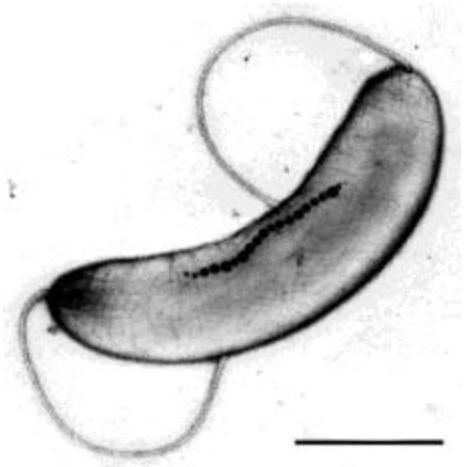


Figure 15.11.2.4: Magnetotaxis. The electron micrograph (courtesy of R. B. Blakemore and N. Blakemore) is of the bacterium **Magnetospirillum magnetotacticum** (the bar represents 1 μm.). The row of dark objects within the cell are particle of magnetite ($FeO{\cdot}Fe_2O_3$). These act like a compass needle, and in this species, which was found in a freshwater pond in New Hampshire (USA), orient their owner toward the North Pole.

Magnetotactic bacteria in the Southern Hemisphere achieve the same result by swimming toward the South Pole. Particles resembling these magnetite particles have been found in the Martian meteorite ALH84001. If they were not formed by a purely chemical process (as some believe), they would be another entry in the hunt for evidence that Mars can (or could) support life.

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15.11.3: Learned Behavior

Habituation

Habituation is a reduction in a previously-displayed response when no reward or punishment follows. If you make an unusual sound in the presence of the family dog, it will respond usually by turning its head toward the sound. If the stimulus is given repeatedly and nothing either pleasant or unpleasant happens to the dog, it will soon cease to respond. This lack of response is not a result of fatigue or sensory adaptation and is long-lasting; when fully habituated, the dog will not respond to the stimulus even though weeks or months have elapsed since it was last presented.

Sensitization

Sensitization is an **increase** in the response to an innocuous stimulus when that stimulus occurs after a punishing stimulus.

Sensitization in sea slugs

When the siphon of the sea slug **Aplysia** is gently touched, the animal withdraws its gill for a brief period. However, if preceded by an electrical shock to its **tail**, the same gentle touch to the siphon will elicit a longer period of withdrawal. The sensitization response to a single shock (blue bar) dies out after about an hour, and returns to baseline after a day (yellow). So it is an example of short-term memory.

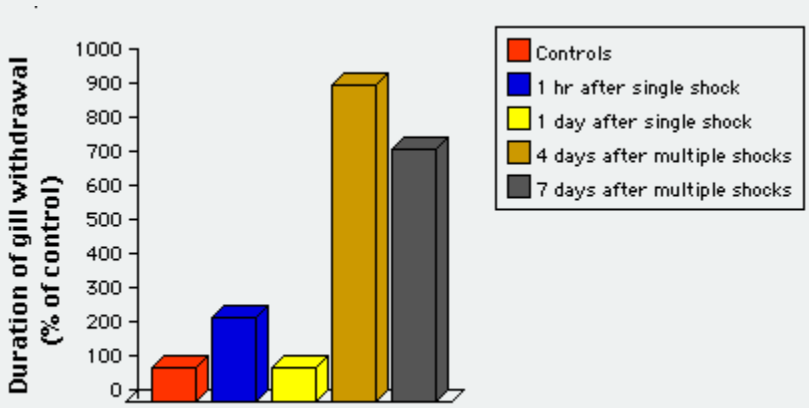


Figure 15.11.3.1 Sensitization in a sea slug

However, if the animal is sensitized with multiple shocks given over several days, its **subsequent** response to a gentle touch on the siphon is much larger and is retained longer (tan and gray bars). This is an example of long-term memory and requires protein synthesis. (These findings are the work of Eric R. Kandel, who was awarded a Nobel Prize in 2000.)

Imprinting

If newly-hatched geese are exposed to a moving object of reasonable size and emitting reasonable sounds, they will begin to follow it just as they would normally follow their mother. This is called **imprinting**. The time of exposure is quite critical. A few days after hatching, imprinting no longer occurs. Prior to this time, though, the results can be quite remarkable. A gosling imprinted to a moving box or clucking person will try to follow this object for the rest of its life. In fact, when the gosling reaches sexual maturity, it will make the imprinted object - rather than a member of its own species - the goal of its sexual drive.

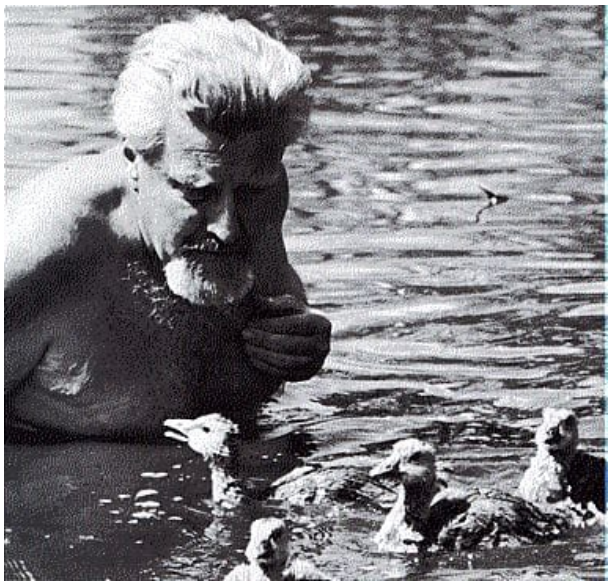


Figure 15.11.3.2 Konrad Lorenz and his imprinted goslings

Much of our knowledge of imprinting was learned from the research of Konrad Lorenz, shown here with some of his imprinted goslings. Lorenz shared a Nobel Prize in 1973 for his discoveries. (Photo by Tom McAvoy; courtesy of LIFE Magazine, ©1955, Time, Inc.)

Male **mice** become imprinted with the odor of littermates during the first three weeks of life. When they reach sexual maturity, they avoid mating with close relatives. The odor is controlled by the major histocompatibility complex (MHC).

The Conditioned Response (CR)

The conditioned response is probably the simplest form of learned behavior. It is a response that as a result of experience comes to be caused by a stimulus different from the one that originally triggered it. The Russian physiologist Ivan Pavlov found that placing meat powder in a dog's mouth would cause it to salivate.

The meat powder, an **unconditioned stimulus** (**US**), triggers a simple inborn reflex involving taste receptors, sensory neurons, networks of interneurons in the brain, and autonomic motor neurons running to the salivary glands thus producing an **unconditioned response** (**UR**).

Pavlov found that if he rang a bell every time he put the meat powder in the dog's mouth, the dog eventually salivated upon hearing the bell alone. This is the **conditioned response** (**CR**). The dog has learned to respond to a substitute stimulus, the **conditioned stimulus** (**CS**).

We assume that the physiological basis of the conditioned response is the transfer, by appropriate neurons, of nervous activity in the auditory areas of the brain to the motor neurons controlling salivation. This involves the development and/or strengthening of neural circuits, which - we may also assume - is characteristic of all forms of learning.

The conditioned response has proved to be an excellent tool for determining the sensory capabilities of other animals. For example, honeybees can be conditioned to seek food on a piece of blue cardboard. By offering other colors to a blue-conditioned bee, Karl von Frisch (who shared the 1973 Nobel Prize with Lorenz) found that honeybees can discriminate between yellow-green, blue-green, blue-violet, and ultraviolet.

Instrumental Conditioning

Pavlov's dogs were restrained and the response being conditioned (salivation) was innate. But the principles of conditioning can also be used to train animals to perform tasks that are not innate. In these cases, the animal is placed in a setting where it can move about and engage in different activities. The experimenter chooses to reward only one, e.g., turning to the left. By first rewarding (e.g., with a pellet of food) even the slightest movement to the left and then only more complete turns, a skilled

experimenter can in about 2 minutes train a naive pigeon to make a complete turn. A little more work and the pigeon will pace out a figure eight.

In the example shown here, the pigeon - presented with two spots of light - pecks at the brighter and reaches down to pick up the grain of food that is its reward.



Figure 15.11.3.3 Instrumental conditioning on pigeons. (Photos courtesy of Roy DeCarava and **Scientific American**.)

Such training is known as **instrumental conditioning** or **operant conditioning**. The latter term was coined by B. F. Skinner, whose skill with the technique enabled him to train pigeons to play ping-pong and even a toy piano! It is also called **trial-and-error** learning because the animal is free to try various responses before finding the one that is rewarded.

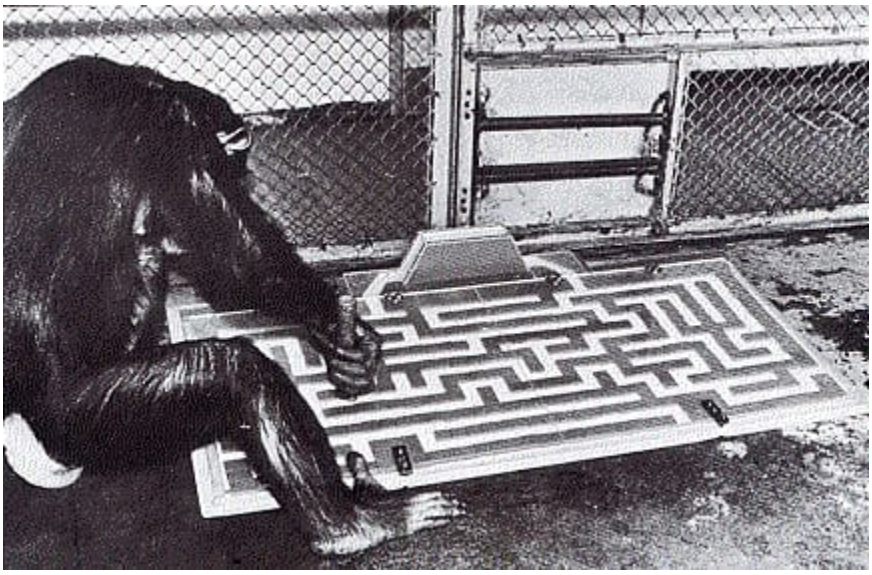


Figure 15.11.3.4 Chimpanzee solving a maze with a magnet

Maze problems are a form of instrumental conditioning in which the animal is faced with a sequence of alternatives.

In this photo (Courtesy of B. Rensch), Julia, a chimpanzee, uses a magnet to move an iron ring through a maze. Julia is able to solve mazes like this on her first attempt most (86%) of the time and sometimes faster than biology students can!

Concepts

Although most animals solve mazes and other problems by trial and error, Julia (and biology students) usually make only one or two random attempts at solving a problem and then, all of a sudden, "get it". They have made an abstract generalization about the specific problem; that is, have formed a **concept**. **Oddity problems** are an example. This young rhesus monkey has learned that food will be found - not under any particular object - but under whichever object is different from the others.

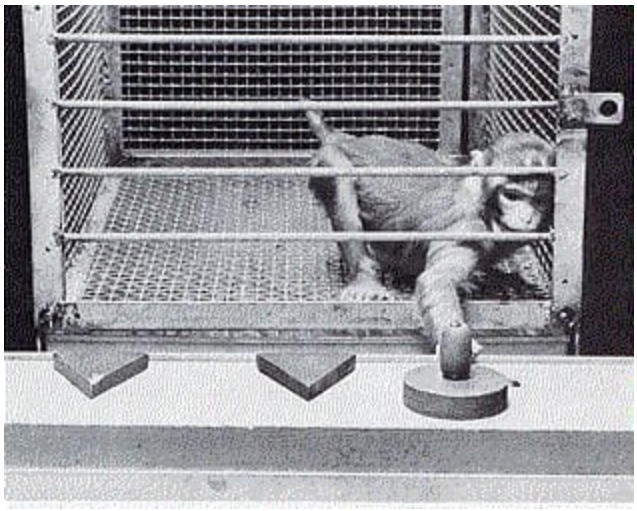


Figure 15.11.3.5 Oddity problems (Photo courtesy of H. F. Harlow, University of Wisconsin Primate Laboratory.)

In monkeys (and probably humans as well), concept formation depends on activity in the prefrontal cortex of the brain. Recent research suggests that honeybees can also solve simple oddity problems!

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15.11.4: Long-Term Potentiation (LTP)

All learning depends on memory. The formation of memories appears to occur in two separate phases, first *short-term memory*. (e.g., Humans undergoing electroshock treatment (to alleviate their depression) are unable to remember events that occurred just prior to the treatment, but their memory of earlier events is unimpaired) that followed by formation of *long-term memory*. Damage to the temporal lobes of the brain can result in the loss of the ability to remember new learning for more than about an hour. Two systems that have been particularly useful in working out the cellular and molecular basis for memory formation are sensitization in the sea slug Aplysia and the study of long-term potentiation (LTP).

Long-Term Potentiation (LTP)

Rats and mice can be trained to solve simple tasks. For example, if a mouse is placed in a pool of murky water, it will swim about until it finds a hidden platform to climb out on. With repetition, the mouse soon learns to locate the platform more quickly. Presumably it does so with the aid of visual cues placed around the perimeter of the pool because it cannot see or smell the platform itself.

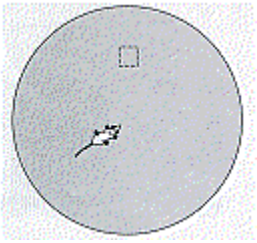


Figure 15.11.4.1 Mouse in murky water

Rats or mice who have had a part of their brain called the **hippocampus** damaged, cannot learn this task, although they continue to solve it quickly if they were trained before their brain damage. This suggests that neurons in the hippocampus are needed for this type of learning. In contrast to the rest of the brain, new neurons are produced in the hippocampus throughout life. They arise from a pool of stem cells in the brain. The integration of newly-formed neurons into existing hippocampal circuitry facilitates the learning of new memories (as well as the forgetting of old ones).

Demonstrating Long-Term Potentiation

The behavior of certain synapses in the "CA1" region of the hippocampus of the rat (or mouse) is consistent with their being essential for this form of long-term memory. Slices of the hippocampus can be removed and its CA1 neurons studied in vitro with recording electrodes. Rapid, intense stimulation of presynaptic neurons evokes action potentials in the postsynaptic neuron. This is just what we would expect from the properties of synapses.

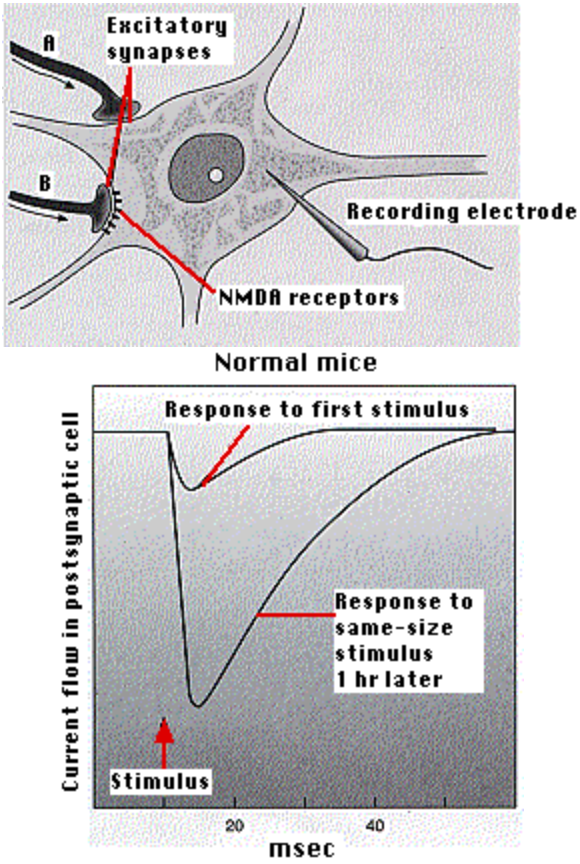


Figure 15.11.4.2 Synapse B response after intense stimulation

What is remarkable about this system is that over time these synapses become increasingly sensitive so that a constant level of presynaptic stimulation becomes converted into a larger postsynaptic output (graph on right). This long-term potentiation can last for weeks. Treatment of a slice of hippocampus with a drug called aminophosphonovaleric acid ("APV") blocks the formation of LTP. This is because APV blocks the action of NMDA receptors, a subset of postsynaptic receptors that normally respond to the excitatory neurotransmitter glutamate (Glu). NMDA receptors (synapse B above) are distinguished from other Glu-activated receptors in being stimulated by the drug, *N*-methyl-D-aspartate (NMDA).

NMDA receptors contain a transmembrane channel that allows for the facilitated diffusion of calcium ions (Ca^{2+}) across the plasma membrane of the synapse. Binding of Glu (or NMDA) and D-serine released from a nearby astrocyte to these receptors opens the channel allowing Ca^{2+} to flow in if — and only if — the same postsynaptic cell has been simultaneously depolarized by other synapses on it (synapse A above). (The drawing is vastly-oversimplified: each CA1 neuron has tens of thousands of synapses on it.)

The influx of Ca^{2+} into the neuron activates an enzyme called calcium-calmodulin-dependent kinase II (CaMKII). Kinases attach phosphate groups to proteins and, in so doing, alter their functioning. In this case, CaMKII phosphorylates a second type of Glu receptor called AMPA receptors, which makes them more permeable to sodium ions (Na^{+}) thus lowering the resting potential of the cell and making it more sensitive to incoming impulses. In addition, there is evidence that the activity of CaMKII increases the number of AMPA receptors at the synapse.

The ability to make transgenic mice has provided tools to test this model of LTP.

Mutant mice

Mice that are homozygous for a mutant CaMKII transgene fail to develop LTP. This was shown (by A. J. Silver, et. al., in **Science 257:**206, 1992) in two ways:

- by measuring the current in the postsynaptic cell of normal mice and mutant mice. The graph above(left) shows that mutant mice do not develop the increase in current flow that normal mice (graph above) do.
- The same failure of LTP occurs when the mice are tested on the hidden platform (graph right).

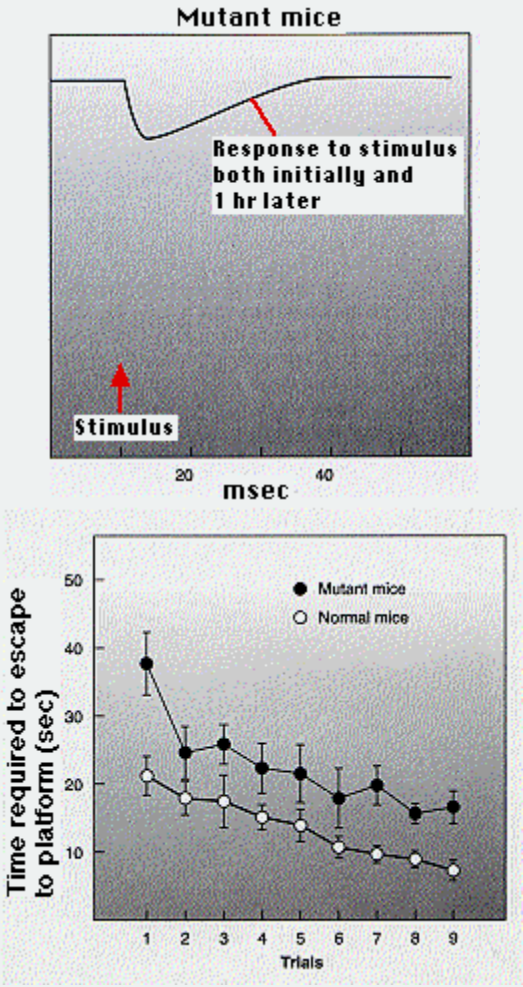


Figure 15.11.4.3 Mutant mice response to stimulus

Transgenic mice

Transgenic mice that make extra-large amounts of NMDA receptors show **enhanced LTP** as shown by

- greater postsynaptic currents in their hippocampus
- their enhanced performance on the hidden-platform test
- and enhanced performance in other tests of learning and memory

These findings were reported in the 2 September 1999 issue of **Nature** by Tang, Y-P, et al. The experiments described above show that manipulations that affect the postsynaptic electrical response (EPSPs) of neurons stimulated electrically also affect learned behavior. They do not show that learning **induces** increased EPSPs in postsynaptic neurons of the hippocampus. Now, researchers at MIT have done just that.

They used rats in which they implanted an array of closely-spaced recording electrodes in the CA1 region of the hippocampus. These rats were then placed in a training apparatus where they learned in a single trial that moving from a lighted chamber to a dark one would give them a shock.

> In just 30 minutes some, but never all, of the recording electrodes picked up increased EPSPs in the CA1 neurons and the number of AMPA receptors increased in the CA1 cells. So learning this conditioned response produced the electrical and synaptic changes of LTP but only in certain regions of the hippocampus. Presumably other types of learning would produce LTP in other parts of the CA1 region. (You can read the report of their work in Whitlock, J. R., *et al*., **Science**, 25 August 2006.)

Early LTP vs. Late LTP

LTP occurs in two phases:

- an early one (in the first hour or so) which involves increased sensitivity of the synapse without any new gene transcription or mRNA translation occurring
- a late one which requires new gene transcription and mRNA translation and results in an increase in the **number** of AMPA receptors accompanied by an increase in the size of the synaptic connection. These changes persist for many hours and even many days. However, increased AMPA receptor formation seems to require continuous stimulation because (in rats, at least) interfering with the process erases late LTP (and memory) even a month later.

This is yet more evidence that memories are acquired in two phases; early and late. Late LTP may involve not only the addition of AMPA receptors to existing synapses but the formation of entirely new synapses. Researches in Geneva, Switzerland have demonstrated that formation of LTP in rat brains coincides with the formation of additional synapses (at least one more) between the presynaptic axon terminal and the dendrite it synapses with. (Report by Toni, N., et al, **Nature**, 25 Nov 99). Presumably this, too, increases the efficiency of synaptic transmission.

Summary

- Rapid, intense stimulation of CA1 neurons in the hippocampus depolarizes them.
- Binding of Glu and D-serine to their **NMDA receptors** opens them.
- Ca^{2+} ions flow into the cell through the NMDA receptors and bind to **calmodulin**.
- This activates calcium-calmodulin-dependent kinase II (**CaMKII**).
    - CaMKII phosphorylates AMPA receptors making them more permeable to the inflow of Na^{+} ions and thus increasing the sensitivity of the cell to depolarization.
    - In time CaMKII also increases the number of AMPA receptors at the synapse.
- Increased gene expression (i.e., protein synthesis — perhaps of AMPA receptors) also occurs during the development of LTP.
- Enlargement of the synaptic connections and perhaps the formation of additional synapses occur during the formation of LTP.

LTP has also been demonstrated in neurons of the cerebellum.

Long-Term Depression (LTD)

Slow, weak electrical stimulation of CA1 neurons also brings about long-term changes in the synapses, in this case, a reduction in their sensitivity. This is called long-term depression or LTD. It reduces the number of AMPA receptors at the synapse. Long-term depression also occurs in isolated preparations of neurons from the sea slug, Aplysia and the cerebellum of mice during the development of an conditioned response (CR)

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15.11.5: Honeybee Navigation

The domestic honeybee, ***Apis mellifera***, is a colonial insect living in hives containing one queen — a fertile female, a few drones (males), and thousands of workers (infertile females). The workers are responsible for keeping the hive clean, building the wax combs of the hive, tending the young and, when they get older (and when their *for* gene gets turned on), and **foraging** for food - nectar and pollen.

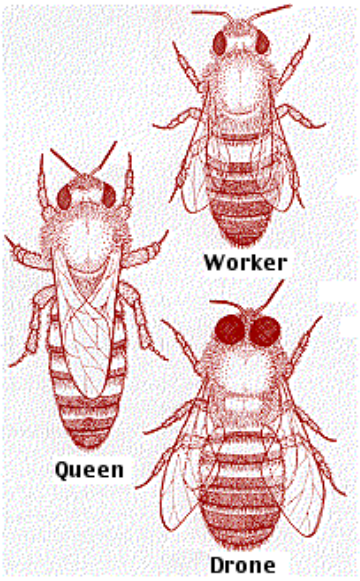


Figure 15.11.5.1 Honeybee castes

ome 5–25% of the workers in the hive are **scouts**. Their job is to search for new sources of food for the other workers, the **foragers**, to harvest. While both scouts and foragers look alike, recent research suggests that they represent stable subpopulations with distinctive patterns of gene expression in their brains. (See Liang, Z. S., *et al.*, in the 9 March 2012 issue of **Science**.)

When food is discovered by scouts, they return to the hive. Shortly after their return, many foragers leave the hive and fly directly to the food. The remarkable thing about this is that the foragers do not follow the scouts back. The scouts may remain in the hive for hours and those that leave continue to hunt for new sources of food even though the foragers are continuing to bring back ample supplies of food from the sites the scouts discovered earlier. So the scout bees have communicated to the foragers the necessary information for them to find the food on their own.

It turns out that the scouts can convey to the foragers information about

- the odor of the food
- its direction from the hive
- its distance from the hive

Distance

When food is within 50–75 meters of the hive, the scouts dance the **"round dance"** on the surface of the comb (left). But when the food is farther than 75 meters from the hive, the scouts dance the **"waggle dance"** (right). The waggle dance has two components: (1) a straight run - the direction of which conveys information about the **direction** of the food and (2) the speed at which the dance is repeated which indicates how far away the food is.

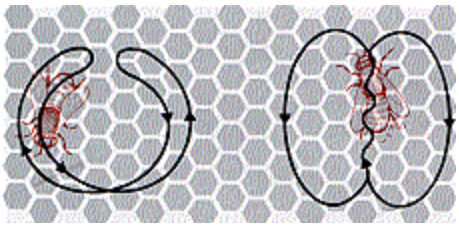


Figure 15.11.5.2 Round Dance

The graph shows the relationship between the speed of the dance and the distance to the food. It is based on data collected by the German ethologist **Karl von Frisch**. It was he who discovered much of what we know today about honeybee communication (and was honored with a Nobel Prize in 1973).

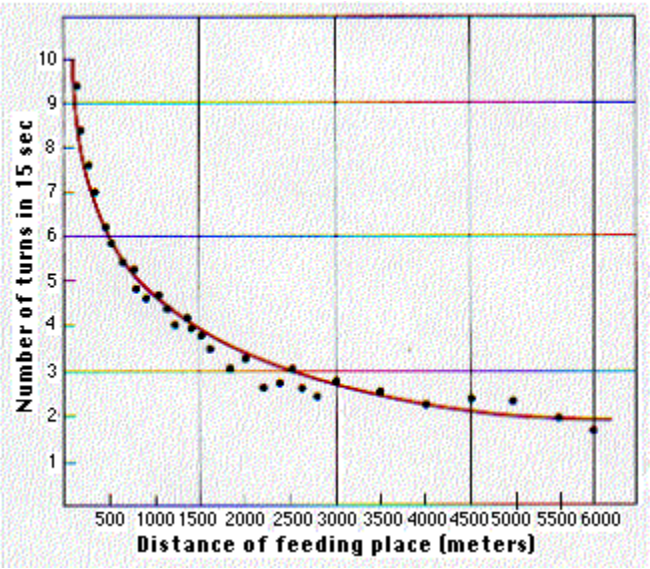


Figure 15.11.5.3 von Frisch graph

How do the bees calculate distance? von Frisch thought they measured the distance by assessing the amount of energy it took to get there. But the mechanism turns out to be quite different. The bees measure distance by the motion of images received by their eyes as they fly.

Flicker Effect

Honeybees, like all insects, have compound eyes. These give little information about depth but are very sensitive to "flicker effect". It has long been known to bee keepers that honeybees respond better to flowers

- moving in the breeze
- with complex petals

The importance of flicker effect can be demonstrated by training honeybees to visit food placed on cards with patterns. For example, the bees can distinguish any figure in the top row from any figure in the bottom row more easily than they can distinguish between any of the figures in either row.

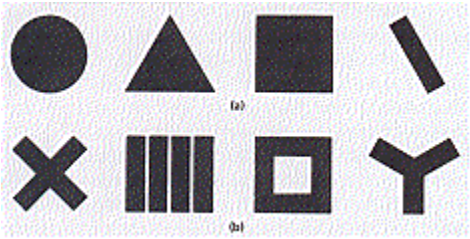


Figure 15.11.5.4 Flicker patterns

Evidence of distance measuring

Working at the University of Notre Dame (Indiana), Esch and Burns found that when the hive and food were placed on top of tall (50 m) buildings, the speed of the waggle dance indicated a distance only half of the distance indicated by bees travelling the same distance (230 m) at ground level. Features of the scenery pass the retina faster when near than when far (compare the changes in your view from an airliner at cruising altitude and as it approaches the runway).

Tests of Searching Behavior

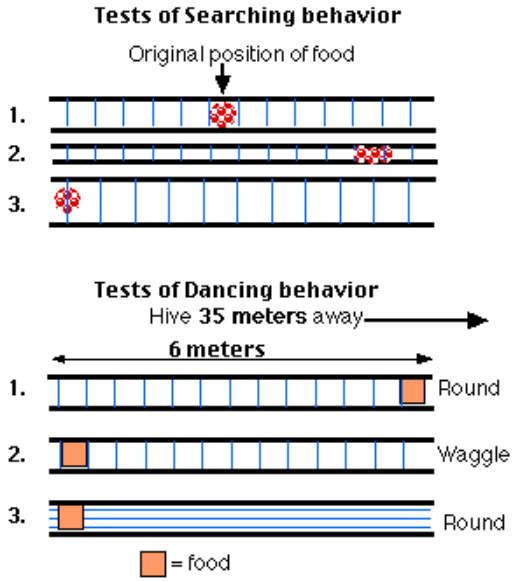


Figure 15.11.5.5 Searching and Dancing behavior in bees

Working at the Australian National University, Srinivasan and his colleagues built tunnels decorating the interior walls with patterns to create flicker. In these three experiments, the bees were first fed in the middle of the tunnel of standard diameter. Then the food was removed.

1. Using the same tunnel, foragers came to same spot in the tunnel.
2. Using a narrower tunnel, foragers began searching for food near its entrance. Forced to fly closer to the vertical stripes, the images moved by faster.
3. Using a tunnel with a larger diameter, foragers searched for food at its far end. Flying farther from the stripes, the images moved by more slowly.

Tests of Dancing Behavior

All tunnels were 6 meters long and located 35 meters from the hive — well within the distance (50–75 m) that normally elicits the **round** dance.

1. Bees were fed at the entrance. Back at the hive, they danced the **round** dance as you would expect.
2. Bees fed at end of tunnel. Back at the hive, they danced the **waggle** dance. Even though the total distance from the hive (41 m) was still well within the "round" range, the complexity of the scenery passed in the last 6 m, elicited the waggle dance.
3. Bees fed at end of tunnel decorated with horizontal stripes. These created no flicker as the bees flew, and on their return to the hive they danced the **round** dance.

You can read about this second set of experiments in Srinivasan, et. al., **Science**, 4 February 2000.

Recruits respond to the misinformation given by returning scouts

More recently, the Esch and Srinivasan groups have teamed up to show that naive foragers are fooled by the misinformation that the returning scouts give them. When scouts were fed only 11 m from the hive but at the far end of a striped tunnel, they danced

the waggle dance back at the hive as though the food had been 70 m away. Most of the workers they recruited flew out (bypassing the tunnel) 70 m looking for food. You can read about these experiments in Ungless, et. al., **Nature**, 31 May 2001.

Direction

By itself, the knowledge that food is 6 kilometers (3.7 miles) away is not very useful. But von Frisch also noted that the direction of the straight portion of the waggle dance varied with the direction of the food source from the hive and the time of day.

- At any one time, the direction changes with the location of the food.
- With a fixed source of food, the direction changes by the same angle as the sun during its passage through the sky.

But

- The sun is not visible within the hive.
- The scouts dance on the **vertical** surface of the combs.

How, then, do they translate flight angles in the darkened hive?

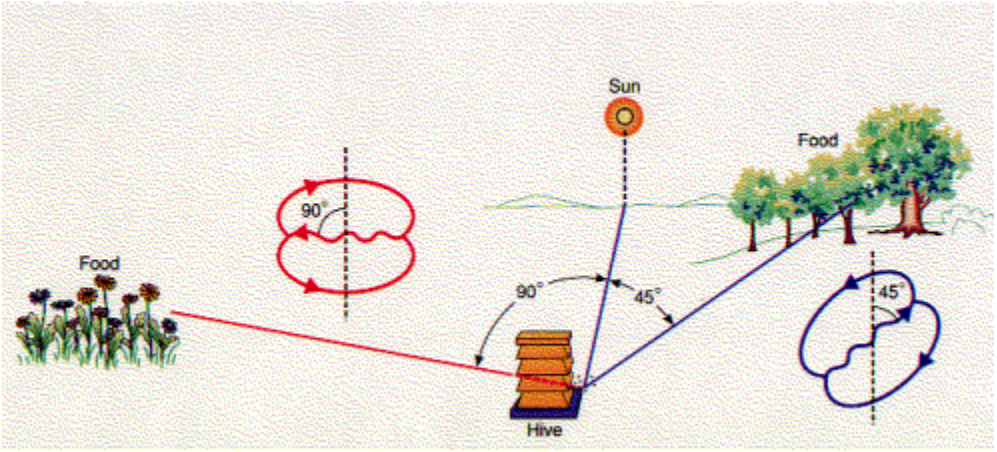


Figure 15.11.5.6 Bee dance

The picture shows the relationship between the angle of the dance on the vertical comb and the bearing of the sun with respect to the location of food. When the food and sun are in the same direction, the straight portion of the waggle dance is directed upward. When the food is at some angle to the right (blue) or left (red) of the sun, the bee orients the straight portion of her dance at the same angle to the right or left of the vertical.

Using radar to track individual bees recruited by the waggle dance, Riley, J. R., *et al*., (**Nature**, 12 May 2005) have shown that the recruits do fly in the indicated direction. They even adjust their flight path to compensate for being blown off course by the wind. However, their course is seldom so precise that they can find the food without the aid of vision and/or smell as they neared it.

Other features

Time Sense

When scouts remain in the hive for a long period, they shift the direction of the straight portion of the waggle dance as the day wears on (and the direction of the sun shifts). But they cannot see the sun in the darkened hive. Evidently, they are "aware" of the passing time and make the necessary corrections.

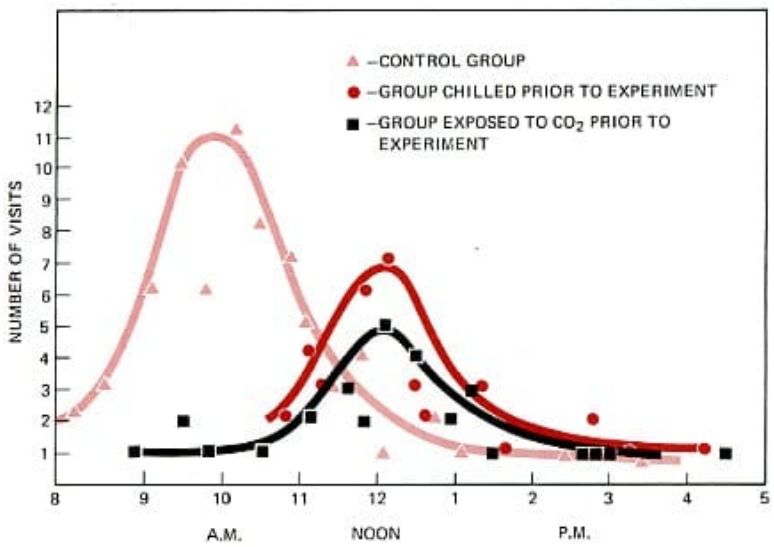


Figure 15.11.5.7 Time Sense

The time sense of honeybees has long been known to people who have sweet snacks in their garden at a set time every day. Within minutes of the regular time, foraging bees arrive for their share of the jam. The speed of the bee's clock seems to be related to its metabolic rate. If normally punctual bees are chilled (to lower their metabolic rate) or exposed to an anesthetizing concentration of carbon dioxide they arrive late to the picnic table (graph above).

Polarized light

von Frisch also discovered that scouts (and foragers) don't actually have to see the sun to navigate. As long as they can see a small patch of clear blue sky, they get along fine. This is because sky light is partially polarized, and the plane of polarization in any part of the sky is determined by the location of the sun. Try it by rotating a pair of polaroid® sun glasses!

Swarming

Before a new queen emerges, the old queen leaves the hive, taking many of the workers with her. The swarm usually settles somewhere, e.g., on a tree branch, while scouts go searching for a new home. Each scout that finds a promising site, returns to the swarm and dances on it just as though she had found food. Eventually, the swarm departs for the location promoted most vigorously. Once the new hive is established, many of the scouts that found the site will become food scouts.

Grooming

Workers have another type of dance — rapidly vibrating from side to side — that tells other workers that she needs help removing dust, pollen, etc. from hard-to-reach places on her body.

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15.11.6: Avoiding Predation

Most animals get their food from preying on other organisms, and much of the life of animals involves eating and avoiding being eaten. So it is not surprising to find many examples of adaptations that increase the effectiveness of predation and minimize the risk of being preyed upon. We shall examine some of the devices that help their owners avoid being eaten.

Camouflage (cryptic coloration)

Many animals are patterned to blend in with their surroundings. Some examples include the peppered moth and the winter flounder.

Masquerade

Masquerading animals resemble some inanimate object and thereby escape detection by potential predators (or prey).



Figure 15.11.6.1 Twig caterpillar

The motionless **twig caterpillar** shown here (courtesy of Muriel V. Williams) complete with "buds" and "lenticels" escapes detection by birds (but pays for its cleverness by occasionally having some other insect lay eggs on it by mistake).

Chemical Defenses

Many plants and animals use repellent chemicals to deter predation. Millipedes secrete hydrocyanic acid when disturbed. Some beetles squirt potential predators with such mixtures as 85% acetic acid or 40% formic acid. The discharge of the skunk is another familiar example.But what if you have a powerful defensive weapon but no potential predator notices until it has launched an attack? One solution to this dilemma is the evolution of warning coloration (also called **aposematic coloration**).

Aposematic Coloration



Figure 15.11.6.2 Monarch larva

This is the larva of the monarch butterfly; an example of aposematic coloration. There is no question of camouflage here. Rather this creature is advertising its presence. The milkweed leaves on which it is feeding contain cardiac glycosides that are toxic to vertebrates because they block the activity of the Na^+/K^+ ATPase that is essential for many cell functions. The larva stores these within its body and thus becomes unpalatable to vertebrate predators. The chemicals remain in the body even after metamorphosis, so that adults are unpalatable as well.

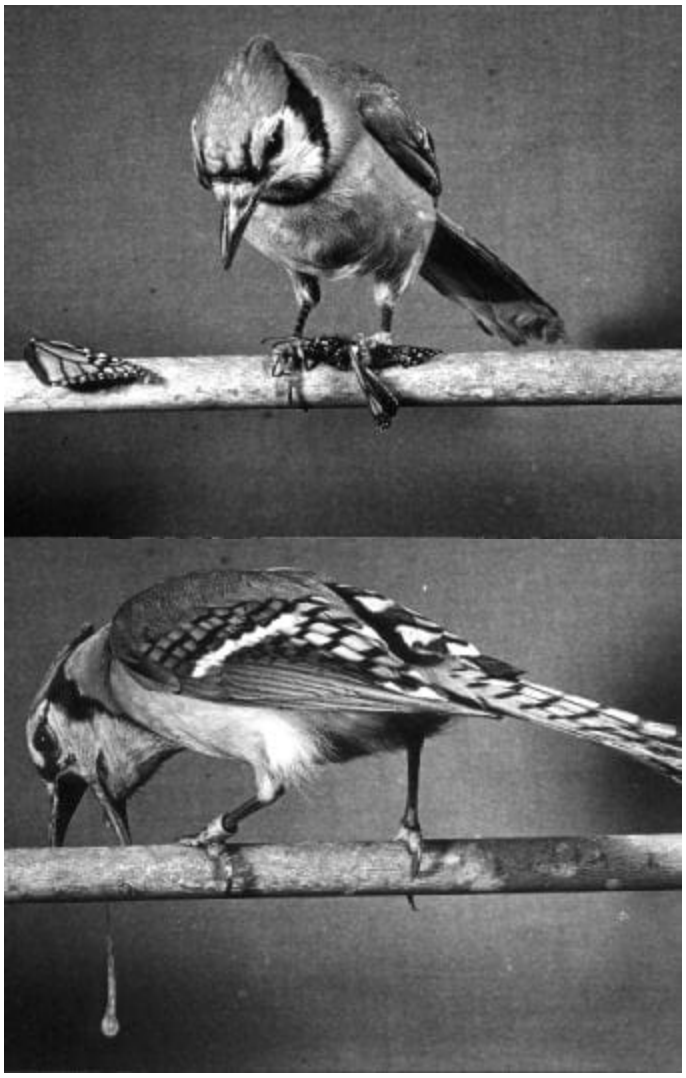


Figure 15.11.6.3 Blu jay

In these photographs (provided by Lincoln P. Brower) a blue jay eats a portion of a monarch butterfly (left) that had fed (in its larval stage) on poisonous milkweed. A short time later, the blue jay vomits (right). Following this episode, the blue jay refused to eat any other monarch offered to it.

Mimicry

Batesian Mimicry

If an animal is not noxious to potential predators, why not look like an animal that is? Some examples:

- A number of harmless snakes closely mimic the bright warning coloration of the **coral snake** — the most poisonous snake in the United States.
- The harmless **robber fly** (right) resembles the **bumblebee** (left) even though the two are not closely related. The robber fly is a dipteran, with only a single pair of wings, while the bumblebee is a hymenopteran with two pairs.
- The **viceroy** butterfly (bottom) contains no toxic substances in its body and presumably is quite palatable (one entomologist declared it tastes like dried toast). If so, the viceroy's striking resemblance to the **monarch** (top) enables it to capitalize on the monarch's unpalatability. (Photos by, and courtesy of, Howard Hoople.)

This phenomenon is called Batesian mimicry (named after Henry W. Bates, a nineteenth-century naturalist who studied many such cases).

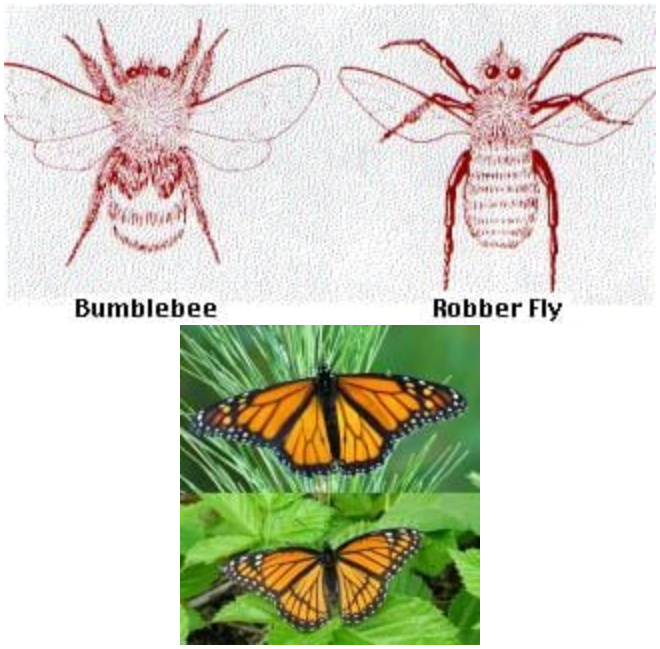


Figure 15.11.6.4 Batesian mimicry in robberfly(left) and civeroy butterfly(right)

Müllerian Mimicry

Some unpalatable animals closely resemble other equally unpalatable species. Such mimicry is called Müllerian mimicry (in honor of the German zoologist Fritz Müller, who studied it). Presumably each species gains a measure of protection from the occasional, but educational, losses of the other species to predators. Lincoln Brower, who has studied the monarch and viceroy, believes that the viceroy is as unpalatable to potential predators as the monarch, and thus is really an example of Müllerian mimicry.

Aggressive Mimicry

Some carnivores have evolved devices with which they mimic the prey (or potential mate) of other (usually smaller) predators. They use these devices as lures.

Two examples:

- The angler fish (Antennarius) displays a lure resembling a small fish. The lure is a development of the spine of the first dorsal fin. This species of anglerfish, which was found in the Philippines, is 9.5 cm long. Note its use of camouflage: its texture (and color) closely resemble the sponge- and algae-encrusted rocks found in its habitat.

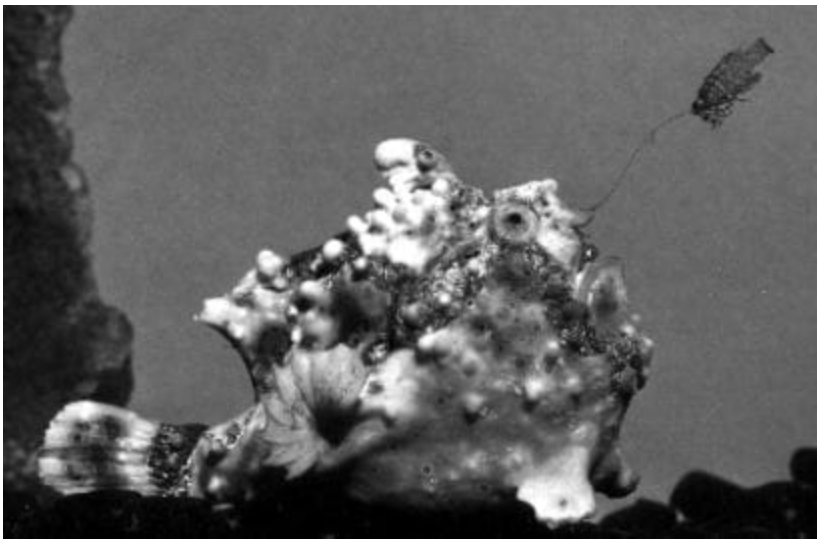


Figure 15.11.6.5 Angler fish courtesy of David B. Grobecker from Pietsch, T. W. and D. B. Grobecker, Science, 201:369, 1978.)

Fireflies use their flashes to attract mates. The pattern differs from species to species. In one species, the females sometimes mimic the pattern used by females of another species. When the males of the second species respond to these "femmes fatales", they are eaten!

Group Behavior

Cooperation between members of a social species often reduces the severity of predation. Grazing ungulates are usually organized so that the stronger are on the outside of the herd, the weaker within.



Figure 15.11.6.6 Musk oxen

In this photo (courtesy of Ted Grant, National Film Board of Canada), musk oxen have responded to a threat by forming a circle with the females and young in the center. Both herds of ungulates and flocks of birds often have members who are extra-watchful, ready to give the alarm if danger threatens. When alarmed, smelt (a kind of fish) release a pheromone into the water that warns other members of the school. When a honeybee stings an intruder, she release isoamyl acetate, which excites other bees to join the attack.

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15.11.7: Pheromones

Pheromones are chemicals released by an organism into its environment enabling it to communicate with other members of its own species.

Pheromones in Insects

Alarm Pheromone: When an ant is disturbed, it releases a pheromone that can be detected by other ants several centimeters away. They are attracted by low concentrations of the pheromone and begin to move toward the region of increasing concentration. As they get nearer to their disturbed nestmate, their response changes to one of alarm. The higher concentration causes them to run about as they work to remedy the disturbance. Unless additional amounts of the alarm pheromone are released, it soon dissipates. This ensures that once the emergency is over, the ants return quietly to their former occupations. Honeybees also have an alarm pheromone (which is a good thing not to elicit around a colony of "Africanized" bees).

Trail Pheromone: Certain ants, as they return to the nest with food, lay down a trail pheromone. This trail attracts and guides other ants to the food. It is continually renewed as long as the food holds out. When the supply begins to dwindle, trailmaking ceases. The trail pheromone evaporates quickly so other ants stop coming to the site and are not confused by old trails when food is found elsewhere. And at least in one species of ant, trails that no longer lead to food are also marked with a repellant pheromone.



Figure 15.11.7.1: Trail pheromone

A stick treated with the trail pheromone of an ant (left) can be used to make an artificial trail which is followed closely by other ants emerging from their nest (right). The trail will not be maintained by other ants unless food is placed at its end. (Photos courtesy of Sol Mednick and Scientific American).

Queen Pheromone: Honeybee queens spend their lives literally surrounded by a retinue of worker bees. The queen emits a pheromone that is a complex mixture of unsaturated hydrocarbons, fatty acids, and other organic molecules. Among its effects:

- inducing the workers to feed and groom her
- inhibiting the workers from building queen cells and rearing new queens
- inhibiting ovary development in the workers

Sex Attractants

Hundreds of pheromones are known with which one sex (usually the female) of an insect species attracts its mates. Many of these sex attractants or their close chemical relatives are available commercially. They have proved useful weapons against insect pests in two ways:

- **Male Confusion**: Distributing a sex attractant throughout an area masks the insect's own attractant and thus may prevent the sexes getting together. This "communication disruption" has been used successfully against a wide variety of important

pests. For example, the sex attractant of the cotton boll weevil has reduced the need for conventional chemical insecticides by more than half in some cotton-growing areas.

- **Insect Monitoring**: Insect sex attractants are also valuable in monitoring pest populations. By baiting traps with the appropriate pheromone, a build-up of the pest population can be spotted early. Even if a conventional insecticide is the weapon chosen, its early use reduces
  - the amount needed
  - damage to the crop
  - cost to the grower
  - possible damage to the environment.

  Early detection of pest build-up is a key ingredient in the system known as integrated pest management (IPM).



Figure 15.11.7.2 Gypsy moth

The photo (courtesy of USDA) shows the feathery antennae of a male gypsy moth. These detect the pheromone released by the females (who do not fly). In some insects, a single molecule of sex attractant is enough to elicit a response.

Sexual Deception

- By an animal: Many species of spiders prey exclusively on moths of certain species and only on the males. Studies of one species of spider, *Mastophora cornigera*, show that it releases a mixture of volatile compounds that mimic the sex pheromone of the moth species it preys upon. Male moths flying upwind in search of a female end up eaten instead!
- By a plant: A number of species of orchids - each pollinated by the males of a single species of insect (wasps or bees) — emit the same pheromone that is the sex attractant by which females of the insect species attract the males for mating.

Pheromones in Mammals

Releaser Pheromones: Many mammals (e.g., dogs and cats) deposit chemicals in and/or around their "territory". As these vaporize, they signal to other members of the species of the presence of the occupant of the territory. Domestic rabbit mothers release a mammary pheromone that triggers immediate nursing behavior by their babies (pups). A good thing, too, as mothers devote only 5–7 minutes a day to feeding their pups so they had better be quick about it.

Many animals, including mammals, signal with alarm pheromones. Although neither the source nor the chemical nature of alarm pheromones are known in any mammal, stressed animals release something that triggers quick behavior (e.g., flight or fight) in other members of their species. The pheromone is detected in a special cluster of cells located at the very tip of the nose and thus in a position to detected airborne molecules even before the vomeronasal organ (see below) or nasal epithelium can. The detectors on these cells are primary cilia.

Primer Pheromones: Rats and mice give off pheromones that elicit mating behavior. However, the response is not immediate as it is in the releaser pheromones of mother rabbits and insects. Instead, detection of the pheromone primes the endocrine

system of the recipient to make the changes, e.g., ovulation, needed for successful mating. Primer pheromones are detected by the olfactory epithelium with which normal odors are detected and also in most mammals (but not humans) by the *vomeronasal organ (VNO)*. The VNO is a patch of receptor tissue in the nasal cavity distinct from the olfactory epithelium. The receptors are G-protein-coupled transmembrane proteins similar to those that mediate olfaction, but encoded by entirely different genes. The neurons leading from the VNO take a separate path into and through the brain.

Human pheromones: It has long been noticed that women living close together (e.g., college roommates) develop synchronous menstrual cycles. This is thought to be because they release two (as yet uncharacterized) primer pheromones: (1) one prior to ovulation that tends to speed up the onset of ovulation in others and (2) one after ovulation that tends to delay the onset of ovulation in other women. Both pheromones are released from the armpits. The pheromones are not detected consciously as odors, but presumably trigger the hormonal changes that mediate the menstrual cycle.

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15.11.8: Circadian Rhythms in Drosophila and Mammals

This page explores circadian rhythms in eukaryotes and some microbes, highlighting daily cycles in gene expression and behavior controlled by light-activated clock proteins. In Drosophila, distinct clocks assist in adapting to seasonal changes, influencing behaviors like reproduction. Mammalian rhythms are regulated by a master clock in the suprachiasmatic nucleus, demonstrating parallels across species.

All eukaryotes and some microbes (e.g., cyanobacteria) display changes in gene activity, biochemistry, physiology, and behavior that wax and wane through the cycle of days and nights.

Examples:

- The level of the hormone melatonin that rises in your body during the night and falls during the day.
- Fruit flies (***Drosophila melanogaster***) hatch in greatest numbers just at dawn.

Even when the organism is placed in constant conditions (e.g., continuous darkness), these rhythms persist. However, without environmental cues, they tend to be somewhat longer or somewhat shorter than 24 hours giving rise to the name **circadian** rhythms (L. *circa* = about; *dies* = day).

The genetics and molecular biology of circadian rhythms have been studied in several model organisms including

- some unicellular eukaryotes
- fungi
- plants (**Arabidopsis**)
- invertebrates (**Drosophila**)
- mammals (mice, rats, and humans)

What has emerged are some remarkable similarities in mechanisms across these various groups. Let us take a detailed look at the mechanism in Drosophila.

The Circadian Clock in Drosophila

A number of genes in Drosophila are turned on when the animal is exposed to light:

- effector genes whose products mediate the animal's responses (e.g. hatching or molting)
- **clock genes** whose products regulate the circadian clock. Two key members of this group are:
  - **period** (***per***)
  - ***timeless*** (***tim***)

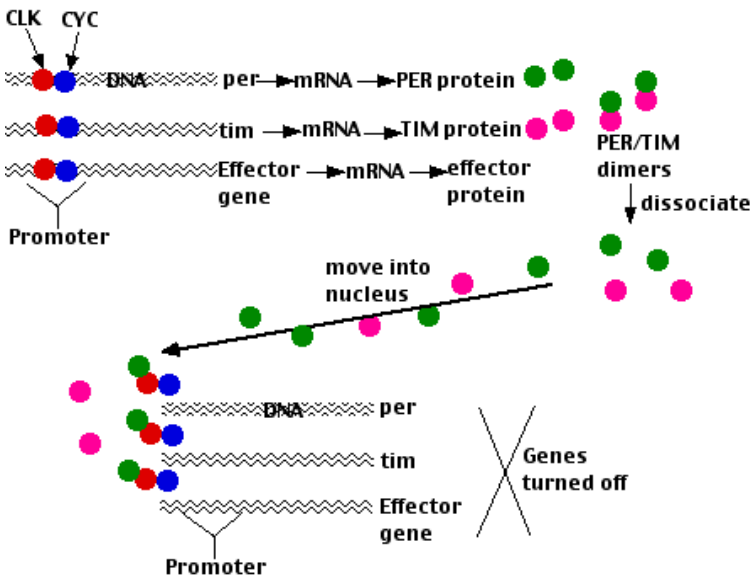


Figure 15.11.8.1 Promoter

Activation of all of these genes requires that their promoters are bound by the protein transcription factors

- **CLOCK** encoded by the gene ***clock*** (***clk***)
- **CYCLE** encoded by the gene ***cycle*** (***cyc***)

(The names of proteins will be designated with capitalized Roman letters; the genes that encode them indicated in lower case italics.)

The Mechanism

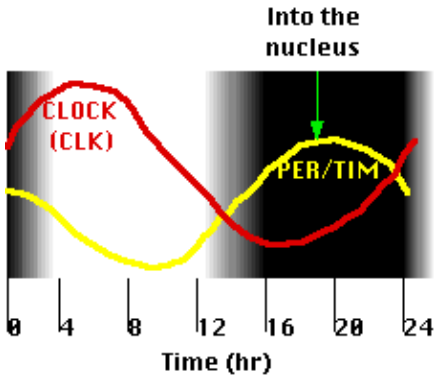


Fig.15.11.8.2 Mechanism

- The **PER** and **TIM** proteins (synthesized on ribosomes in the cytoplasm) form dimers.
- When the concentration of these gets high enough (early evening), they dissociate and are transported into the nucleus.
- Here **PER**
  - binds to the **CLK/CYC** transcription factors, removing them from the promoters of the genes they activate; thus shutting off transcription. Because these genes include ***per*** and ***tim***, the result is a negative feedback loop; that is, the product of the ***per*** gene inhibit its own synthesis (as well as that of *tim*). Just as the heat of a furnace turns through the thermostat - its own production off, so a rising level of PER/TIM dimers turns off further synthesis of them. As the level then falls, this inhibition is lifted and PER/TIM activity begins anew.
  - turns on ***clock*** gene expression.
- The time required for the different effects results in the levels of **PER/TIM** and **CLOCK** oscillating in opposite phases with a circadian (~24 hr) rhythm (figure).

Setting the Clock

Even without any external cues (e.g., alternating light and dark), the cycles persist although they tend to drift away from environmental time.

- Under natural conditions, the clocks are precise.
- This is because they are "set" (synchronized) by environmental cues, of which light is one of the most important.

In Drosophila, it works like this.

- Light (blue) is absorbed by the protein cryptochrome (**CRY**).
- This causes an allosteric change in its conformation enabling it to bind to **TIM** and **PER**.
- This causes TIM and PER to break down (in proteasomes) ending their inhibition of gene transcription.
  - If this happens when **PER/TIM levels are rising** (late in the "day"), it sets the clock back.
  - If it happens when **PER/TIM levels are declining** (late in the "night"), it sets the clock ahead.

The Circadian Clock in Mammals

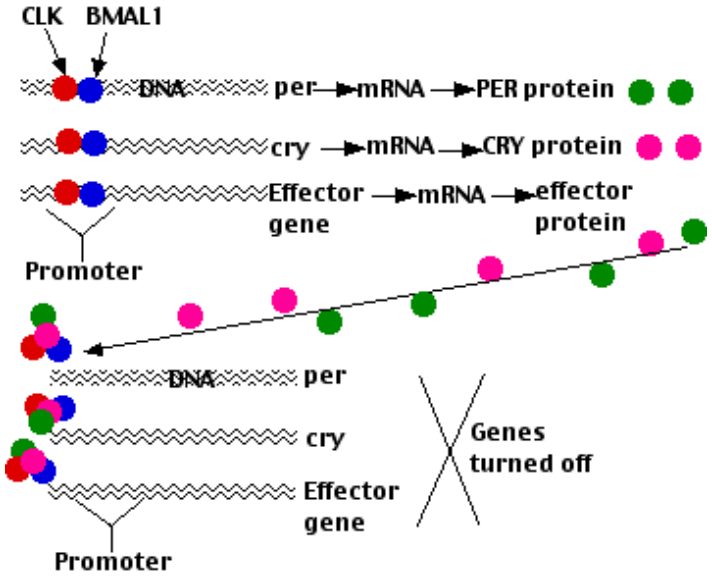


Figure 15.11.8.3 Clock in mammals

The circadian clock in mammals resembles that in Drosophila in a number of ways with many of the participating genes being homologous. However, there are some differences:

- The transcription factors that turn **on** the light-induced promoters are dimers of the **CLOCK** protein and a protein designated **BMAL1**. These dimers turn **on**
  - three **Per** genes
  - two ***Cry*** genes, the genes encoding cryptochrome
  - hundreds of **effector genes** whose products control a wide variety of metabolic functions (e.g., cellular respiration, glycolysis, gluconeogenesis, lipid metabolism)
- The **PER** and **CRY** mRNAs are exported to the cytoplasm where they are translated.
- The **PER** and **CRY** proteins then enter the nucleus where they inhibit CLOCK-BMAL1 thus turning OFF transcription of *Per* and *Cry*, and are then degraded in proteasomes.

In due course these actions allow CLOCK and BMAL1 to once again stimulate transcription of *Per* and *Cry*. Thus this negative feedback loop causes the levels of BMAL1 and PER/CRY to oscillate in opposite phases (as CLOCK and PER/TIM do in Drosophila).

Many tissues in mammals, e.g., liver, skeletal muscle, and the beta cells of the pancreas have endogenous clocks. But all of these are under the control of a **"master clock"**, the **suprachiasmatic nucleus** (**SCN**) - clusters of neurons in the hypothalamus.

The blood levels of many hormones have strong circadian rhythms. Examples:

- hormones synthesized in the hypothalamus, e.g. **vasopressin**
- whose secretion is controlled by the hypothalamus such as growth hormone and cortisol
- insulin

Setting the Clock

By light

Mice who are totally blind (lacking both rods and cones) have no trouble keeping their circadian clock on time.

They are able to do this because

- Some 1–2% of the ganglion cells in their retina - instead of depending on signals arriving from rods and/or cones detect light directly.
- These ganglion cells have an extensive network of dendrites that contain the pigment **melanopsin**.
- When exposed to light (diffuse light is fine), these ganglion cells become depolarized and send their signals back to the suprachiasmatic nucleus (SCN).

By food

In mice, the SCN clock, set by light/dark cycles, is the master clock as long as food is available all the time (the normal situation in the laboratory). However, if food is offered for only a 4-hour period when the mice would normally be asleep, they shift several circadian activities so that, for example, once a day they begin running about just before they expect food to be given to them. This rhythm continues even if the mice are kept in constant darkness.

The clock mechanism is the same as the light/dark-driven clock in the SCN, but the machinery that sets the clock by food is located in a different part of the hypothalamus, the dorsomedial hypothalamic nuclei (**DMH**).

Mice with both copies of the *Bmal1* gene knocked out, are unable to establish circadian rhythms to either light or food. However, injections of an adeno-associated virus vector (AAV) containing the *Bmal1* gene

- into the SCN restores the light clock but not the food-set clock
- into the DMH restores the food but not the light-set clock.

Sleep Disorders

Unlike mice, people who are totally blind cannot set the clock in their SCN. As a result, their circadian rhythm drifts out of phase with the actual cycle of day and night. These people often are bothered by feeling sleepy during the day and wide awake when they want to be asleep at night. A report in the 12 October 2000 issue of the **New England Journal of Medicine** tells of a group of blind people who were able to set their clocks with the help of a dose (10 mg) of melatonin at bedtime. However, this treatment worked only when the subject's circadian rhythm had drifted so that the normal rise in melatonin from the pineal gland was occurring in the early evening; that is, the dose of melatonin had to be given when it could boost the endogenous level of the hormone.

Some people suffer from a disorder called **familial advanced sleep-phase syndrome** (FASPS). As the name suggests, it is inherited ("familial") and their circadian clocks run fast ("advanced"). Those afflicted tend to wake up several (up to four) hours earlier than normal.

One cause of the disorder turns out to be a point mutation in the human **PER2** gene. Exactly how this mutation causes shortening of the circadian cycle is still under investigation.

Photoperiodism

Many plants and animals not only engage in a cycle of daily activities (opening of flowers, waking, feeding, etc.) but also in seasonal activities.

- In plants, such things as production of flowers and making buds dormant in preparation for winter.
- In animals, such things as preparing to migrate and entering and leaving hibernation.

The most reliable clue to the change of season is length of day (temperature is far less reliable!). The farther a plant or animal lives north or south of the equator, the more pronounced the changing ratio of daytime hours to nighttime hours with the changing seasons.

It is not surprising then that both plants and animals mainly depend on photoperiod to prepare for changes in seasonal activities. And what better way to measure the relative length of day and night than by enlisting the machinery by which circadian rhythms are entrained?

As for animals, recent work with Drosophila suggests that this animal uses two circadian clocks to monitor the changing length of day and night.

- An "evening" clock that takes over in the long days of summer.
- A"morning" clock that is inhibited by light but takes over when the nights are getting longer;

The molecular machinery (Cry, Tim, Per, etc.) for each clock is confined to separate neurons in two different parts of the brain.

In these experiments, Drosophila is using the two clocks to adapt daily — not seasonal — cycles of activity to the changing seasons. But this machinery for measuring photoperiod could enable them to prepare for seasonal changes in activity, e.g., to stop forming eggs at the end of the summer. However, other studies examining such seasonal changes in Drosophila find that the photoperiodic response is independent of circadian responses. So we must await more experiments to resolve the question.

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UNIT 16: THE ANATOMY AND PHYSIOLOGY OF PLANTS

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16.1: PLANT ANATOMY

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16.1.5: Arabidopsis Thaliana

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16.1.1: Plant Tissues

This page explains the different types of plant tissues and their roles: meristematic tissue enables growth; protective tissue covers surfaces; parenchyma aids in storage and photosynthesis; sclerenchyma offers support; collenchyma strengthens growth areas; xylem transports water and minerals; and phloem carries sugars. Each tissue type is essential to the overall structure and function of plants.

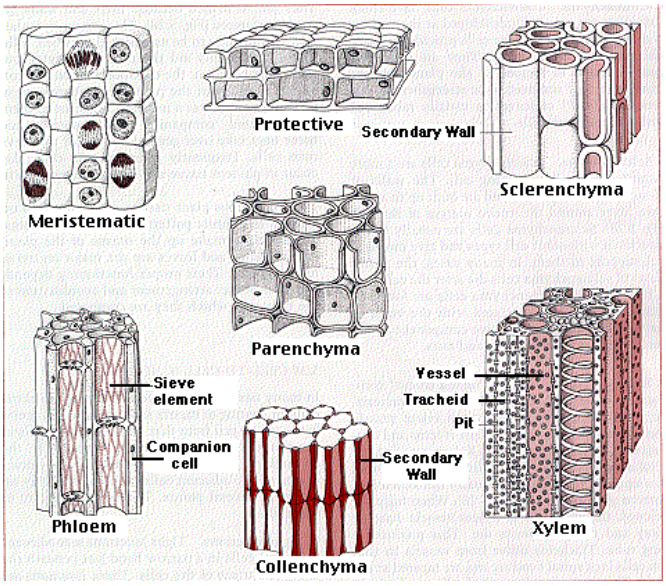


Figure 16.1.1.1 Types of plant tissues

Meristematic

The main function of meristematic tissue is mitosis. The cells are small, thin-walled, with no central vacuole and no specialized features.

Meristematic tissue is located in

- the **apical meristems** at the growing points of roots and stems.
- the **secondary meristems** (lateral buds) at the **nodes** of stems (where branching occurs), and in some plants,
- meristematic tissue, called the **cambium**, that is found within mature stems and roots.

The cells produced in the meristems soon become differentiated into one or another of several types.

Protective

Protective tissue covers the surface of leaves and the living cells of roots and stems. Its cells are flattened with their top and bottom surfaces parallel. The upper and lower **epidermis of the leaf** are examples of protective tissue.

Parenchyma

The cells of parenchyma are large, thin-walled, and usually have a large central vacuole. They are often partially separated from each other and are usually stuffed with plastids. In areas not exposed to light, colorless plastids predominate and food storage is the main function. The cells of the white potato are parenchyma cells. Where light is present, e.g., in leaves, chloroplasts predominate and photosynthesis is the main function.

Sclerenchyma

The walls of these cells are very thick and built up in a uniform layer around the entire margin of the cell. Often, the cell dies after its cell wall is fully formed. Sclerenchyma cells are usually found associated with other cells types and give them mechanical support.

Sclerenchyma is found in stems and also in leaf veins. Sclerenchyma also makes up the hard outer covering of seeds and nuts.

Collenchyma

Collenchyma cells have thick walls that are especially thick at their corners. These cells provide mechanical support for the plant. They are most often found in areas that are growing rapidly and need to be strengthened. The **petiole** ("stalk") of leaves is usually reinforced with collenchyma.

Xylem

Xylem conducts water and dissolved minerals from the roots to all the other parts of the plant.

In angiosperms, most of the water travels in the **xylem vessels**. These are thick-walled tubes that can extend vertically through several feet of xylem tissue. Their diameter may be as large as 0.7 mm. Their walls are thickened with secondary deposits of cellulose and are usually further strengthened by impregnation with **lignin**. The secondary walls of the xylem vessels are deposited in spirals and rings and are usually perforated by pits. Xylem vessels arise from individual cylindrical cells oriented end to end. At maturity the end walls of these cells dissolve away, and the cytoplasmic contents die. The result is the xylem vessel, a continuous nonliving duct.

Xylem also contains **tracheids**. These are individual cells tapered at each end so the tapered end of one cell overlaps that of the adjacent cell. Like xylem vessels, they have thick, lignified walls and, at maturity, no cytoplasm. Their walls are perforated so that water can flow from one tracheid to the next. The xylem of ferns and conifers contains only tracheids.

In woody plants, the older xylem ceases to participate in water transport and simply serves to give strength to the trunk. Wood is xylem. When counting the annual rings of a tree, one is counting rings of xylem.

Phloem

The main components of phloem are **sieve elements** and **companion cells**.

Sieve elements are so named because their end walls are perforated. This allows cytoplasmic connections between vertically-stacked cells. The result is a **sieve tube** that conducts the products of photosynthesis - sugars and amino acids - from the place where they are manufactured (a "source"), e.g., leaves, to the places ("sinks") where they are consumed or stored; such as

- roots
- growing tips of stems and leaves
- flowers
- fruits, tubers, corms, etc.

Sieve elements have no nucleus and only a sparse collection of other organelles. They depend on the adjacent companion cells for many functions. **Companion cells** move sugars, amino acids and a variety of macromolecules into and out of the sieve elements. In "source" tissue, such as a leaf, the companion cells use transmembrane proteins to take up - by active transport - sugars and other organic molecules from the cells manufacturing them. Water follows by osmosis. These materials then move into adjacent sieve elements through plasmodesmata. The pressure created by osmosis drives the flow of materials through the sieve tubes.

In "sink" tissue, the sugars and other organic molecules leave the sieve elements through plasmodesmata connecting the sieve elements to their companion cells and then pass on to the cells of their destination. Again, water follows by osmosis where it may leave the plant by transpiration or increase the volume of the cells or move into the xylem for recycling through the plant.

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16.1.2: Roots

This page describes the structure and functions of plant roots, detailing components like the root tip, root cap, and areas for elongation and differentiation. It discusses water and mineral uptake, emphasizing the importance of root hairs and mycorrhizal fungi. Minerals are absorbed actively, often against concentration gradients, while plants utilize inorganic nutrients directly. Organic fertilizers require breakdown for plant use.

Root Structure

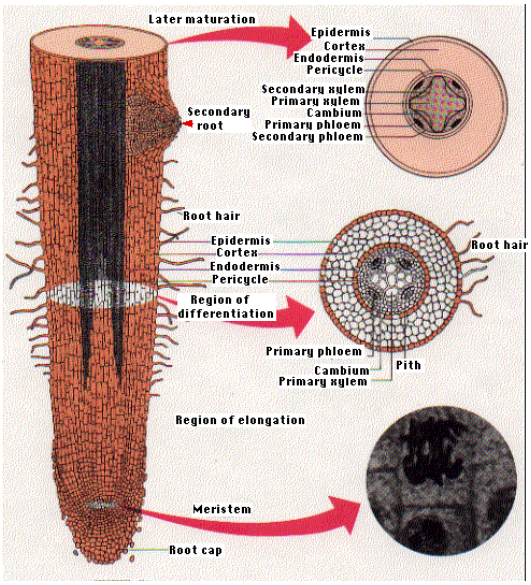


Figure 16.1.2.1 The root

The Root Tip

The root tip consists of a

- root cap — a sheath of cells that
  - protect the meristem from abrasion and damage as the root tip grows through the soil;
  - secrete the growth hormone auxin;
  - detect water and nutrients in the soil;
  - detect gravity and respond with **gravitropism**.
- **meristem** - a region of rapid mitosis, which produces the new cells for root growth.

Because of the frequency of mitosis in the meristem, root tips are often used to demonstrate mitosis in the laboratory The inset is a photo (courtesy of Carolina Biological Supply Co.) of anaphase in the meristem of an onion root tip.

The Region of Elongation

Here the cells produced by mitosis undergo a period of elongation in the direction of the axis of the root.

The Region of Differentiation

Here develop the differentiated tissues of the root.

- Epidermis - A single layer of flattened cells at the surface. When first formed, epidermal cells have extensions — the **root hairs** — which greatly increase the surface area available for the uptake of nutrients from the soil. The photo below shows

the root hairs in the region of differentiation of a germinating radish seed.



Figure 16.1.2.2 Radish root

- **Cortex -** A band of parenchyma cells that develops beneath the epidermis. It stores food. Its inner surface is bounded by a single layer of cells, the
- **Endodermis**
- **Stele**
  - **Pericycle -** the outer boundary of the stele. **Secondary roots** branch from it.
  - **Xylem -** arranged in bundles in a spokelike fashion
  - **Phloem -** alternates with xylem
- **Cambium -** In older parts of the root, another meristem forms between the xylem and phloem. Mitosis in the cambium produces new "**secondary xylem**" to the inside and **secondary phloem** to the outside.

Water Uptake

Water enters the root through the epidermis. Once within the epidermis, water passes through the cortex, mainly traveling between the cells. However, in order to enter the stele, it must pass through the cytoplasm of the cells of the endodermis.

Once within the stele, water is free again to move between cells as well as through them. In young roots, water enters directly into the xylem. In older roots, it may have to pass first through a band of phloem and cambium. It does so by traveling through horizontally-elongated cells, the xylem rays.

Mineral Uptake

One might have expected that minerals would enter the root dissolved in water. But, in fact, minerals enter separately:

- Even when no water is being absorbed, minerals enter freely - mostly through the root hairs.
- Minerals can enter against their concentration gradient; that is, by active transport. For example, plants can take up K^+ from the soil against a ten-thousand-fold concentration gradient; e.g., from as little as 10 µM in the soil to 100 mM in the cell.
- Anything that interferes with the metabolism of root hairs interferes with mineral absorption.
- The root hairs are also the point of entry of mycorrhizal fungi. These transport minerals - especially phosphorus - to the root hair in exchange for carbohydrates from the plant.
- In legumes, the root hairs are the point of entry of rhizobia that will establish the mutualistic partnership enabling the plant to convert atmospheric nitrogen into protein.

Plants absorb their nutrients in inorganic form

For examples:

- nitrogen enters as nitrate (NO_3^-) or ammonium ions (NH_4^+)
- phosphorus as PO_4^{3-}
- potassium as K^+
- calcium as Ca^{2+}

When you hear of the virtues of organic fertilizers, remember that such materials meet no nutritional need of the plant until their constituents have been degraded to inorganic forms. Organic matter does play an important role in making good soil texture, but only to the extent that it can yield inorganic ions can it meet the nutritional needs of the plant.

Once within the epidermis, inorganic ions pass inward from cell to cell, probably through plasmodesmata. The final step from the cytoplasm of the pericycle cells to the xylem is probably accomplished once again by active transport.

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16.1.3: Stems

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This page compares the stem tissue organization of dicots and monocots. Dicots, exemplified by the basswood tree, have three main stem areas: bark for protection, cortex for food storage, and cambium for producing new xylem and phloem. Xylem supports the plant and transports water and minerals.

The organization of the tissues of the stem differs between dicots and monocots.

The Woody Dicot Stem

The drawing shows a sector of a cross section through a 5-year old twig from a basswood tree (Tilia). The stem has three areas:

- bark
- wood
- pith

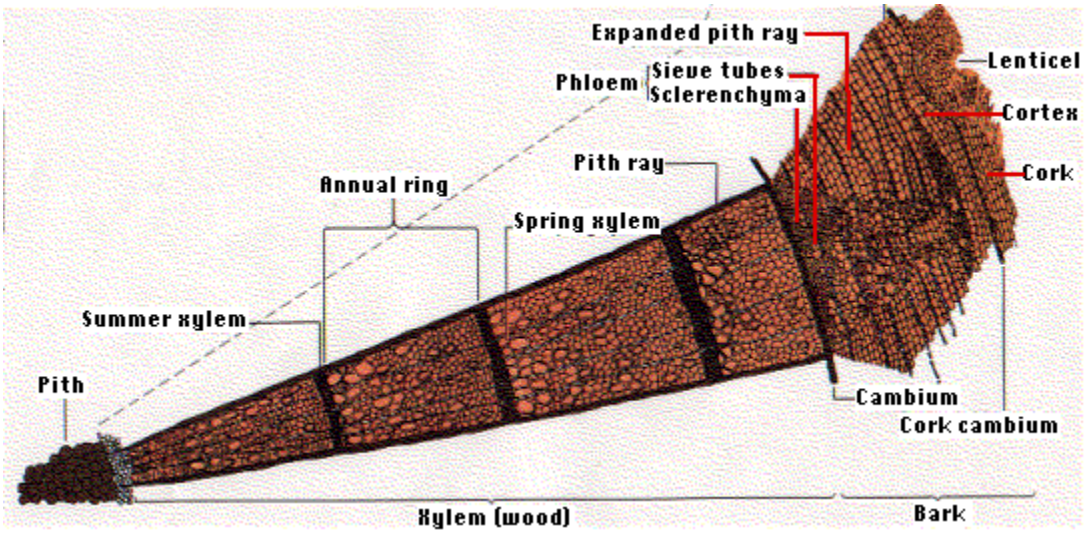


Figure 16.1.3.1 Dicot stem

Bark

- Cork - The outer part of the bark is protected by layers of dead **cork** cells impregnated with suberin. **Suberin** is waxy and cuts down water loss from the stem. But suberin is as impervious to air as it is to water. The gas exchange needs of the living cells beneath the cork are met by openings in the cork called **lenticels**.
- **Cortex -** Layers of parenchyma cells. These store food (as they do in the root). In the very young stem (before cork has formed), they may have chloroplasts and carry on photosynthesis.
- **Cork cambium -** In older stems, a meristem forms between the cork and cortex. Mitosis of its cells produces more cork.
- **Expanded pith rays -** Regions of parenchyma that store food.
- **Phloem -** Bundles of sieve tubes surrounded and supported by sclerenchyma. Translocation of food through the stem takes place in the sieve tubes.

Cambium

During the growing season, mitosis in this band of meristematic tissue produces new phloem to the outside and new xylem to the inside.

Xylem

Xylem makes up the **wood** region. The xylem vessels made in the spring, when water is plentiful, have larger diameters than those made later in the season. No xylem is made during the dormant season. The visual contrast between the late summer xylem of one season and the spring xylem of the next creates the **annual ring**. Xylem serves two functions:

- transport of water and minerals up the stem
- support

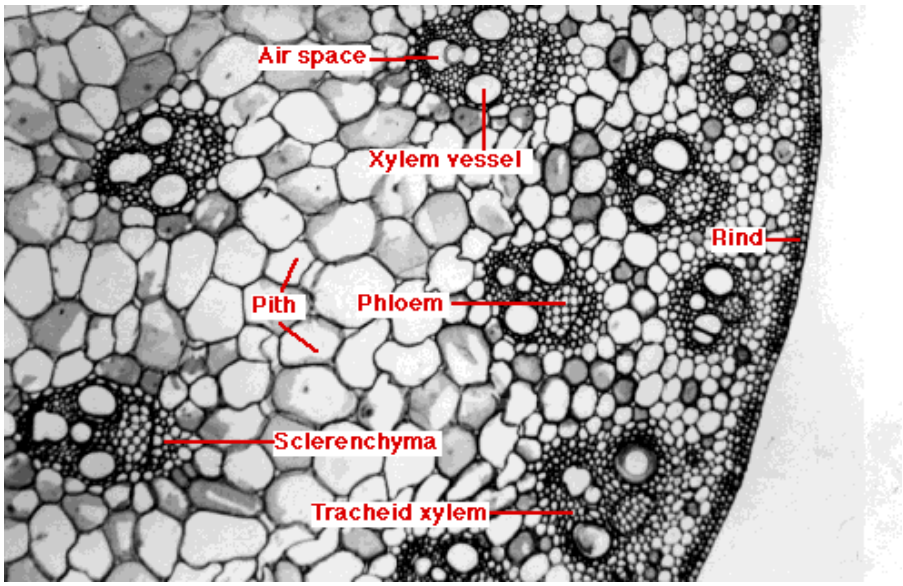


Figure 16.1.3.2 Monocot stem

The photograph (courtesy of Turtox) shows the organization of tissues in the corn (maize) stem, a typical monocot. The corn stem consists of:

- an external **rind**
- an interior filled with **pith**.

Vascular bundles are scattered through the pith.

Each vascular bundle contains:

- a layer of **sclerenchyma** that provides support
- a bundle of phloem containing
  - **sieve tubes** used for food transport
  - their **companion cells**.

- four **xylem vessels**
- a group of **xylem tracheids**
- both carry water and dissolved minerals up the stem

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16.1.4: The Leaf

This page describes the leaf structure, highlighting its layers: the upper epidermis, which reduces water loss, and the palisade layer for photosynthesis; the spongy layer for sugar storage and gas exchange; and the lower epidermis with stomata regulated by guard cells. It also outlines the role of leaf veins, which contain xylem and phloem for water and nutrient supply, and the protective sclerenchyma cells around them, facilitating photosynthesis and food transport.

Layers in a leaf

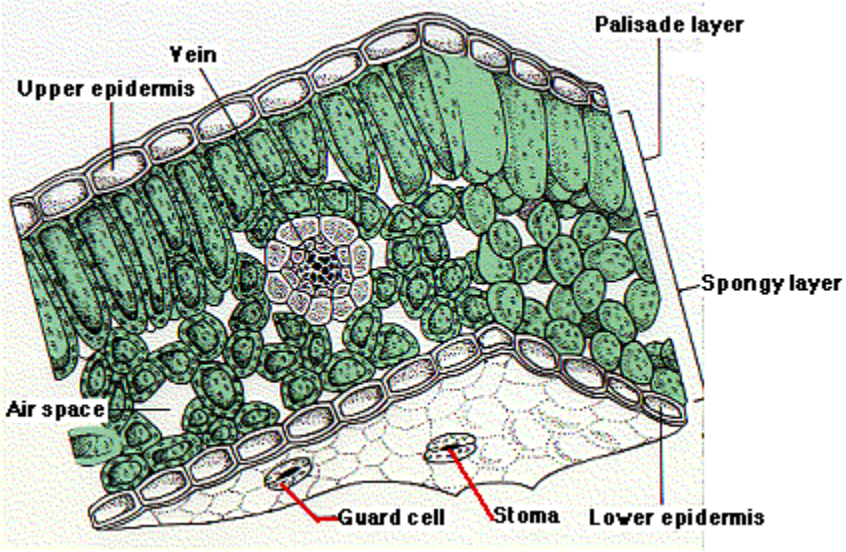


Figure 16.1.4.1 Leaf structure

Upper epidermis

This is a single layer of cells containing few or no chloroplasts. The cells are quite transparent and permit most of the light that strikes them to pass through to the underlying cells. The upper surface is covered with a waxy, waterproof **cuticle**, which serves to reduce water loss from the leaf.

Palisade layer

This consists of one or more layers of cylindrical cells oriented with their long axis perpendicular to the plane of the leaf. The cells are filled with chloroplasts (usually several dozen of them) and carry on most of the photosynthesis in the leaf.

Spongy layer

Lying beneath the palisade layer, its cells are irregular in shape and loosely packed. Although they contain a few chloroplasts, their main function seems to be the temporary storage of sugars and amino acids synthesized in the palisade layer. They also aid in the exchange of gases between the leaf and the environment. During the day, these cells give off oxygen and water vapor to the **air spaces** that surround them. They also pick up carbon dioxide from the air spaces. The air spaces are interconnected and eventually open to the outside through pores called **stomata** (sing., stoma).

Collectively, the palisade and spongy layers make up the **mesophyll**.

Lower epidermis

Typically, most of the stomata (thousands per square centimeter) are located in the lower epidermis. Although most of the cells of the lower epidermis resemble those of the upper epidermis, each stoma is flanked by two sausage-shaped cells called

guard cells. These differ from the other cells of the lower epidermis not only in their shape but also in having chloroplasts. The guard cells regulate the opening and closing of the stomata. Thus they control the exchange of gases between the leaf and the surrounding atmosphere.

Leaf Veins

Not only must the cells of the mesophyll be close to their air supply to secure CO_2 and release O_2 and the reverse in the dark but they must be close to a leaf vein with its

- xylem to supply water and minerals
- phloem to remove synthesized food

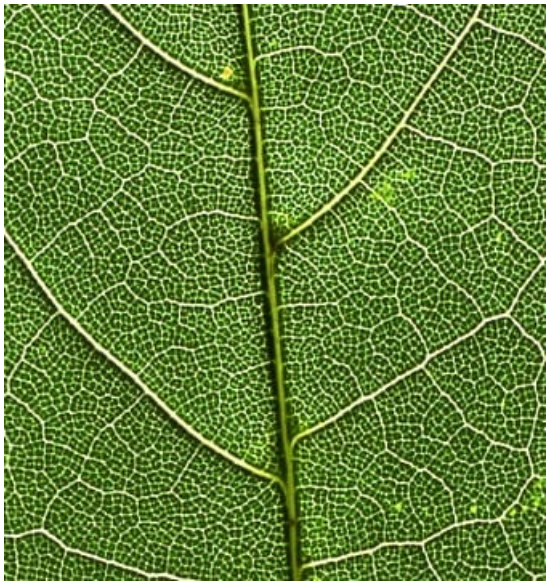


Figure 16.1.4.2 Network of leaf veins

The photo shows the network of leaf veins in a maple leaf. Probably no cell in the spongy layer is more than two cells away from a vein.

The xylem and phloem of veins is often surrounded by layers of sclerenchyma cells. These impart strength to the vein providing a stiff framework to support the soft tissues of the leaf blade.

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16.1.5: Arabidopsis Thaliana

This page discusses Arabidopsis, a model organism in plant biology valued for its small genome, rapid development, and genetic manipulability. Though often seen as a weed, it has been crucial for understanding plant development and genetics through methods like cross-pollination and mutations, leading to significant discoveries in plant functions.



Figure 16.1.5.1 Arabidopsis courtesy of Nicole Hanley Markelz of the Plant Genome Research Outreach Program at Cornell University

This little plant has become to plant biology what *Drosophila melanogaster* and *Caenorhabditis elegans* are to animal biology.

Arabidopsis is an angiosperm, a dicot from the mustard family (Brassicaceae). It is popularly known as thale cress or mouse-ear cress. While it has no commercial value - in fact is considered a weed - it has proved to be an ideal organism for studying plant development.

Some of its advantages as a model organism:

- It has one of the smallest genomes in the plant kingdom: 135 x 10^6 base pairs of DNA distributed in 5 chromosomes (2n = 10) and almost all of which encodes its 27,407 genes.
- Transgenic plants can be made easily using *Agrobacterium tumefaciens* as the vector to introduce foreign genes.
- The plant is small - a flat rosette of leaves from which grows a flower stalk 6–12 inches high.
- It can be easily grown in the lab in a relatively small space.
- Development is rapid. It only takes 5– 6 weeks from seed germination to the production of a new crop of seeds.
- It is a prolific producer of seeds (up to 10,000 per plant) making genetics studies easier.
- Mutations can be easily generated (e.g., by irradiating the seeds or treating them with mutagenic chemicals).
- It is normally self-pollinated so recessive mutations quickly become homozygous and thus expressed.

  Other members of its family cannot self-pollinate. They have an active system of **self-incompatibility**. Arabidopsis, however, has inactivating mutations in the genes - ***SRK*** and ***SCR*** **-** that prevent self-pollination in other members of the family.

- However, Arabidopsis can easily be cross-pollinated to
  - do genetic mapping
  - produce strains with multiple mutations.

Many of the findings about how plants work described throughout these pages were learned from studies with Arabidopsis.

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16.2: PLANT PHYSIOLOGY



- Topic hierarchy

16.2A: Xylem

16.2B: Phloem

16.2C: Transpiration

16.2D: Gas Exchange in Plants

16.2E: Photorespiration and C4 Plants

16.2F: Tropisms

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16.2A: Xylem

Most plants secure the water and minerals they need from their roots. The path taken is:

$$\text{soil} \rightarrow \text{roots} \rightarrow \text{stems} \rightarrow \text{leaves} \tag{16.2A.1}$$

The minerals (e.g., K^{+}, Ca^{2+}) travel dissolved in the water (often accompanied by various organic molecules supplied by root cells), but less than 1% of the water reaching the leaves is used in photosynthesis and plant growth. Most of it is lost in transpiration, which serve two useful functions- it provides the force for lifting the water up the stems and it cools the leaves. Water and minerals enter the root by separate paths which eventually converge in the stele.

The Pathway of Water and Minerals

Soil water enters the root through its epidermis. It appears that water then travels in both the cytoplasm of root cells - called the symplast (i.e., it crosses the plasma membrane and then passes from cell to cell through plasmodesmata) and in the nonliving parts of the root - called the apoplast (i.e., in the spaces between the cells and in the cells walls themselves. This water has not crossed a plasma membrane. However, the inner boundary of the cortex, the endodermis, is impervious to water because of a band of lignified matrix called the casparian strip. Therefore, to enter the stele, apoplastic water must enter the symplasm of the endodermal cells. From here it can pass by plasmodesmata into the cells of the stele. Once inside the stele, water is again free to move between cells as well as through them. In young roots, water enters directly into the xylem vessels and/or tracheids. These are nonliving conduits so are part of the apoplast.

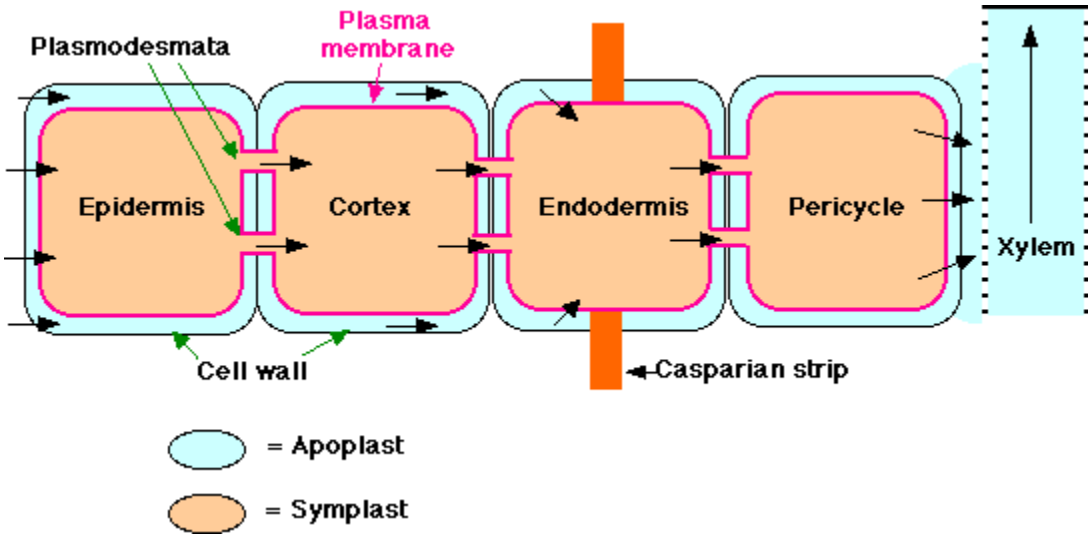


Figure 16.2.1.1: Pathway of water

Once in the xylem, water with the minerals that have been deposited in it (as well as occasional organic molecules supplied by the root tissue) move up in the vessels and tracheids. At any level, the water can leave the xylem and pass laterally to supply the needs of other tissues. At the leaves, the xylem passes into the petiole and then into the veins of the leaf. Water leaves the finest veins and enters the cells of the spongy and palisade layers. Here some of the water may be used in metabolism, but most is lost in transpiration.

Minerals enter the root by active transport into the symplast of epidermal cells and move toward and into the stele through the plasmodesmata connecting the cells. They enter the water in the xylem from the cells of the pericycle (as well as of parenchyma cells surrounding the xylem) through specialized transmembrane channels.

What Forces Water Through the Xylem?

Observations

- The mechanism is based on purely physical forces because the xylem vessels and tracheids are lifeless.
- Roots are not needed. This was demonstrated over a century ago by a German botanist who sawed down a 70-ft (21 meters) oak tree and placed the base of the trunk in a barrel of picric acid solution. The solution was drawn up the trunk, killing nearby tissues as it went.
- However, leaves are needed. When the acid reached the leaves and killed them, the upward movement of water ceased.
- Removing a band of **bark** from around the trunk - a process called **girdling -** does not interrupt the upward flow of water. Girdling removes only the phloem, not the xylem, and so the foliage does not wilt. (In due course, however, the roots - and thus the entire plant - will die because the roots cannot receive any of the food manufactured by the leaves.)

Transpiration-Pull

In 1895, the Irish plant physiologists H. H. Dixon and J. Joly proposed that water is pulled up the plant by **tension** (negative pressure) from above. As we have seen, water is continually being lost from leaves by transpiration. Dixon and Joly believed that the loss of water in the leaves exerts a pull on the water in the xylem ducts and draws more water into the leaf. But even the best vacuum pump can pull water up to a height of only 34 ft (10.4 m) or so. This is because a column of water that high exerts a pressure of ~15 lb/in^2 (103 kilopascals, kPa) just counterbalanced by the pressure of the atmosphere. How can water be drawn to the top of a sequoia (the tallest is 370 feet [113 meters] high)? Taking all factors into account, a pull of at least 270 lb/in^2 (~1.9 x 10^3 kPa) is probably needed.

The answer to the dilemma lies the **cohesion** of water molecules; that is the property of water molecules to cling to each through the hydrogen bonds they form. When ultrapure water is confined to tubes of very small bore, the force of cohesion between water molecules imparts great strength to the column of water. It has been reported that tensions as great as 3000 lb/in^2 (21 x 10^3 kPa) are needed to break the column, about the value needed to break steel wires of the same diameter. In a sense, the cohesion of water molecules gives them the physical properties of solid wires. Because of the critical role of cohesion, the transpiration-pull theory is also called the **cohesion theory**.

support for Cohesion theory

- If sap in the xylem is under tension, we would expect the column to snap apart if air is introduced into the xylem vessel by puncturing it. This is the case.
- If the water in all the xylem ducts is under tension, there should be a resulting inward pull (because of adhesion) on the walls of the ducts. This inward pull in the band of sapwood in an actively transpiring tree should, in turn, cause a **decrease** in the diameter of the trunk.

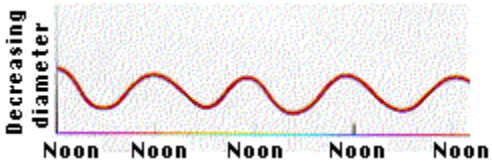


Figure 16.2.1.2 Transpiration in a monterey pine

- The graph shows the results of obtained by D. T. MacDougall when he made continuous measurements of the diameter of a Monterey pine. The diameter fluctuated on a daily basis reaching its **minimum** when the rate of transpiration reached its **maximum** (around noon)
- The rattan vine may climb as high as 150 ft (45.7 m) on the trees of the tropical rain forest in northeastern Australia to get its foliage into the sun. When the base of a vine is severed while immersed in a basin of water, water continues to be taken up. A vine less than 1 inch (2.5 cm) in diameter will "drink" water indefinitely at a rate of up to 12 ml/minute.

  If forced to take water from a sealed container, the vine does so without any decrease in rate, even though the resulting vacuum becomes so great that the remaining water begins to boil spontaneously. (The boiling temperature of water decreases as the air pressure over the water decreases, which is why it takes longer to boil an egg in Denver than in New Orleans.)

- Transpiration-pull enables some trees and shrubs to live in seawater. Seawater is markedly hypertonic to the cytoplasm in the roots of the red mangrove (*Rhizophora mangle*), and we might expect water to leave the cells resulting in a loss in turgor

and wilting. In fact, the remarkably high tensions on the order of 500–800 lb/in^2 (~3 to 5 thousand kPa) in the xylem can pull water into the plant against this osmotic gradient. So mangroves literally desalt seawater to meet their needs.

Problems with the theory

When water is placed under a high vacuum, any dissolved gases come out of solution as bubbles (as we saw above with the rattan vine) - this is called **cavitation**. Any impurities in the water enhance the process. So measurements showing the high tensile strength of water in capillaries require water of high purity - not the case for sap in the xylem. So might cavitation break the column of water in the xylem and thus interrupt its flow? Probably not so long as the tension does not greatly exceed 270 lb/in^2 (~1.9 x 10^3 kPa).

By spinning branches in a centrifuge, it has been shown that water in the xylem avoids cavitation at negative pressures exceeding 225 lb/in^2 (~1.6 x 10^3 kPa). And the fact that sequoias can successfully lift water 358 ft (109 m) - which would require a tension of 270 lb/in^2 (~1.9 x 10^3 kPa) - indicates that cavitation is avoided even at that value. However, such heights may be approaching the limit for xylem transport. Measurements close to the top of the tallest living sequoia (370 ft [=113 m] high) show that the high tensions needed to get water up there have resulted in smaller stomatal openings, causing lower concentrations of CO_2 in the needles, causing reduced photosynthesis, causing reduced growth (smaller cells and much smaller needles). (Reported by Koch, G. W. *et al.*, in *Nature*, 22 April 2004.) So the limits on water transport limit the ultimate height which trees can reach. The tallest tree ever measured, a Douglas fir, was 413 ft. (125.9 meters) high.

Root Pressure

When a tomato plant is carefully severed close to the base of the stem, sap oozes from the stump. The fluid comes out under pressure which is called root pressure. Root pressure is created by the osmotic pressure of xylem sap which is, in turn, created by dissolved minerals and sugars that have been actively transported into the apoplast of the stele.

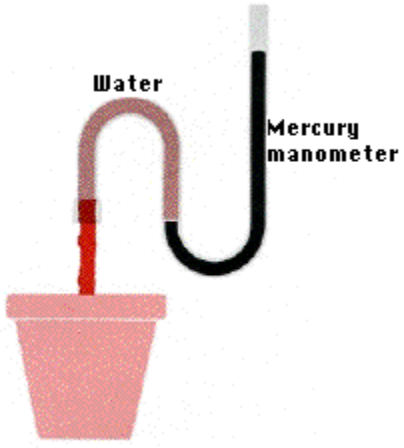


Figure 16.2.1.3: Root pressure

One important example is the sugar maple when, in very early spring, it hydrolyzes the starches stored in its roots into sugar. This causes water to pass by osmosis through the endodermis and into the xylem ducts. The continuous inflow forces the sap up the ducts.

Although root pressure plays a role in the transport of water in the xylem in some plants and in some seasons, it does not account for most water transport.

- Few plants develop root pressures greater than 30 lb/in^2 (207 kPa), and some develop no root pressure at all.
- The volume of fluid transported by root pressure is not enough to account for the measured movement of water in the xylem of most trees and vines.
- Those plants with a reasonably good flow of sap are apt to have the lowest root pressures and vice versa.
- The highest root pressures occur in the spring when the sap is strongly hypertonic to soil water, but the rate of transpiration

is low. In summer, when transpiration is high and water is moving rapidly through the xylem, often no root pressure can be detected.

So although root pressure may play a significant role in water transport in certain species (e.g., the coconut palm) or at certain times, most plants meet their needs by transpiration-pull.

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16.2B: Phloem

Food and other organic substances (e.g., some plant hormones and even messenger RNAs) manufactured in the cells of the plant are transported in the **phloem**. Sugars (usually **sucrose**), amino acids and other organic molecules enter the sieve elements through plasmodesmata connecting them to adjacent **companion cells**. Once within the sieve elements, these molecules can be transported either up or down to any region of the plant moving at rates as high as 110 µm per second.

Two demonstrations:

- **Girdling**. Girdling is removing a band of bark from the circumference of the tree. Girdling removes the phloem, but not the xylem. If a tree is girdled in summer, it continues to live for a time. There is, however, no increase in the weight of the roots, and the bark just above the girdled region accumulates carbohydrates. Unless a special graft is made to bridge the gap, the tree eventually dies as its roots starve.



Figure 16.2.2.0: Girdling, also called ring barking or ring-barking, is the process of completely removing a strip of bark (consisting of Secondary Phloem tissue, cork cambium, and cork) around a tree's outer circumference, causing its death. Here girdling occurs by deliberate human action to give new habitats to species of dead woods (Lille, North of France, Parc de la Cidatelle (Bois de Boulogne). (CC-BY-SA-3.0; Lamiot).

- The pictures below are autoradiographs showing that the products of photosynthesis are transported in the phloem.

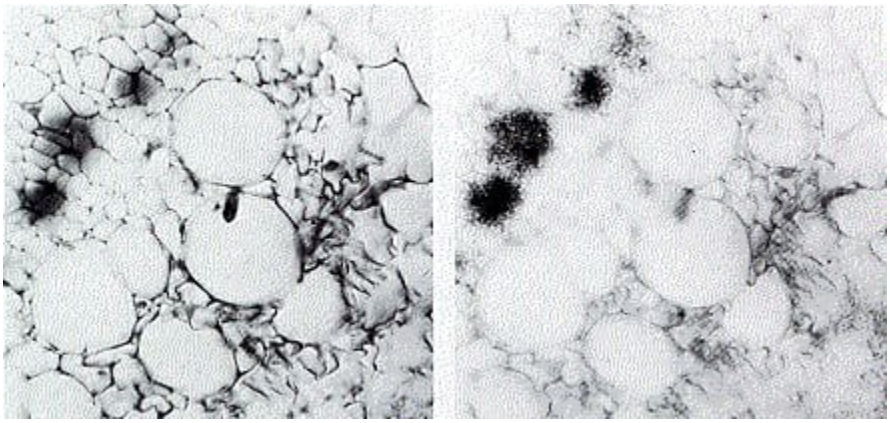


Figure 16.2.2.1 Products of photosynthesis transported in phloem courtesy of R. S. Gage and S. Aronoff

A cucumber leaf was supplied with radioactive water (^{3}HOH) and allowed to carry on photosynthesis for 30 minutes.

Then slices were cut from the petiole of the leaf and covered with a photographic emulsion. Radioactive products of photosynthesis darkened the emulsion where it was in contact with the phloem (upper left in both photos), but not where it was in contact with the xylem vessels (center). In the photomicrograph on the left, the microscope is focused on the tissue in order to show the cells clearly; on the right, the microscope has been focused on the photographic emulsion.

Some fruits, such as the pumpkin, receive over 0.5 gram of food each day through the phloem. Because the fluid is fairly dilute, this requires a substantial flow. In fact, the use of radioactive tracers shows that substances can travel through as much as 100 cm of phloem in an hour.

Mechanism that drives translocation of food through the phloem

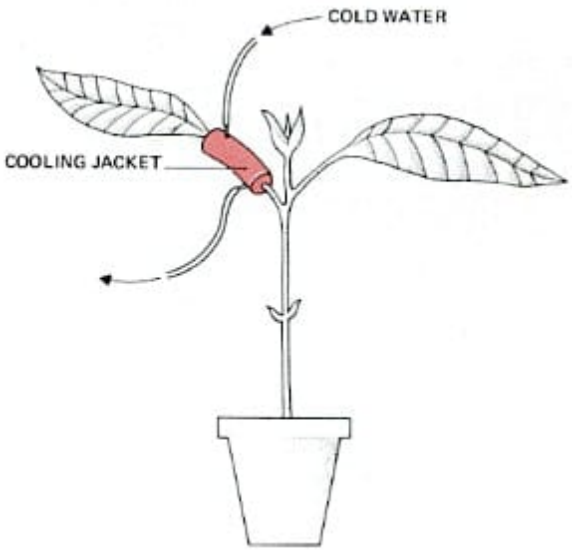


Figure 16.2.2.2 Chilling petiole

Translocation through the phloem is dependent on metabolic activity of the phloem cells (in contrast to transport in the xylem).

- Chilling its petiole slows the rate at which food is translocated out of the leaf (above).
- Oxygen lack also depresses it.
- Killing the phloem cells puts an end to it.

The Pressure-Flow Hypothesis

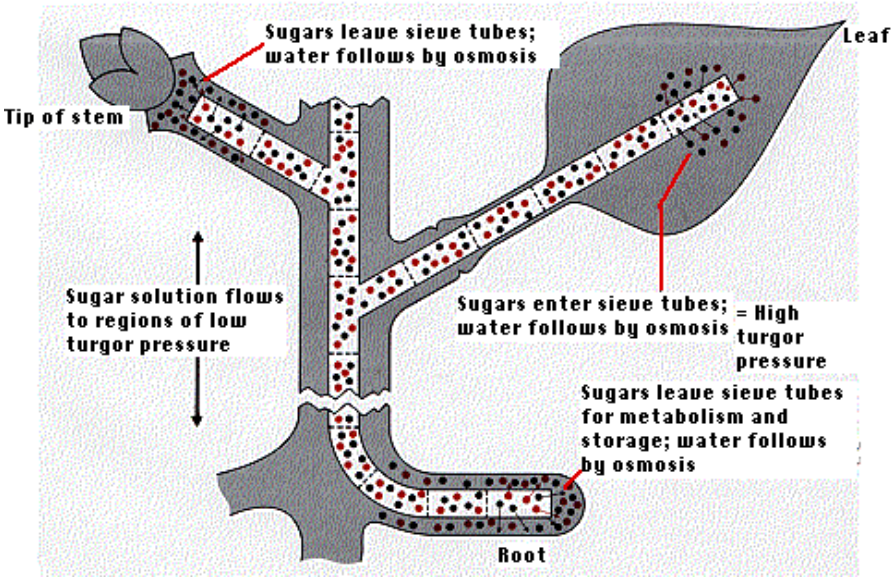


Figure 16.2.2.3 Pressure flow

The best-supported theory to explain the movement of food through the phloem is called the **pressure-flow hypothesis**.

- It proposes that water containing food molecules flows under pressure through the phloem.
- The pressure is created by the difference in water concentration of the solution in the phloem and the relatively pure water in the nearby xylem ducts.
- At their "source" - the leaves - sugars are pumped by active transport into the companion cells and sieve elements of the phloem.
- As sugars (and other products of photosynthesis) accumulate in the phloem, water enters by osmosis.

  In the figure, sugar molecules are represented in black, water molecules in red.)

- Turgor pressure builds up in the sieve elements (similar to the creation of root pressure).
- As the fluid is pushed down (and up) the phloem, sugars are removed by the cortex cells of both stem and root (the "**sinks**") and consumed or converted into starch.
- Starch is insoluble and exerts no osmotic effect.
- Therefore, the osmotic pressure of the contents of the phloem decreases.
- Finally, relatively pure water is left in the phloem, and this leaves by osmosis and/or is drawn back into nearby xylem vessels by the suction of transpiration-pull.

Thus it is the pressure gradient between "source" (leaves) and "sink" (shoot and roots) that drives the contents of the phloem up and down through the sieve elements.

Tests of the theory

1. The contents of the sieve elements must be under pressure.

This is difficult to measure because when a sieve element is punctured with a measuring probe, the holes in its end walls quickly plug up. However, aphids can insert their mouth parts without triggering this response.



Figure 16.2.2.4 Pressure flow hypothesis in reality

Left: when it punctures a sieve element, sap enters the insect's mouth parts under pressure and some soon emerges at the other end (as a drop of honeydew that serves as food for ants and bees).

Right: honeydew will continue to exude from the mouthparts after the aphid has been cut away from them.

2. The osmotic pressure of the fluid in the phloem of the leaves must be greater than that in the phloem of the food-receiving organs such as the roots and fruits. Most measurements have shown this to be true.

Transport of Messenger RNA (mRNA) through the Phloem

Plant scientists at the Davis campus of the University of California (reported in the 13 July 2001 issue of **Science**) have demonstrated that messenger RNAs can also be transported long distances in the phloem. They grafted normal tomato scions onto mutant tomato stocks and found that mRNAs synthesized in the stock were transported into the scions. These mRNAs converted the phenotype of the scion into that of the stock.

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16.2C: Transpiration

Transpiration is the evaporation of water from plants. It occurs chiefly at the leaves while their stomata are open for the passage of CO_2 and O_2 during photosynthesis. But air that is not fully saturated with water vapor (100% relative humidity) will dry the surfaces of cells with which it comes in contact. So the photosynthesizing leaf loses substantial amount of water by evaporation. This transpired water must be replaced by the transport of more water from the soil to the leaves through the xylem of the roots and stem.

Transpiration is not simply a hazard of plant life. It is the "engine" that pulls water up from the roots to:

- supply photosynthesis (1%-2% of the total)
- bring minerals from the roots for biosynthesis within the leaf
- cool the leaf

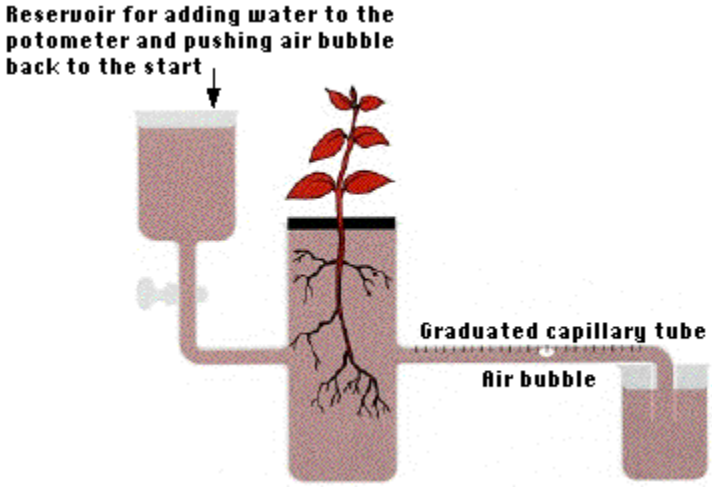


Figure 16.2.3.1 Potometer

Using a potometer (above), one can study the effect of various environmental factors on the rate of transpiration. As water is transpired or otherwise used by the plant, it is replaced from the reservoir on the right. This pushes the air bubble to the left providing a precise measure of the volume of water used.

Environmental factors that affect the rate of transpiration

1. Light
Plants transpire more rapidly in the light than in the dark. This is largely because light stimulates the opening of the stomata (mechanism). Light also speeds up transpiration by warming the leaf.

2. Temperature
Plants transpire more rapidly at higher temperatures because water evaporates more rapidly as the temperature rises. At 30°C, a leaf may transpire three times as fast as it does at 20°C.

3. Humidity
The rate of diffusion of any substance increases as the difference in concentration of the substances in the two regions increases.When the surrounding air is dry, diffusion of water out of the leaf goes on more rapidly.

4. Wind
When there is no breeze, the air surrounding a leaf becomes increasingly humid thus reducing the rate of transpiration. When a breeze is present, the humid air is carried away and replaced by drier air.

5. Soil water

A plant cannot continue to transpire rapidly if its water loss is not made up by replacement from the soil. When absorption of water by the roots fails to keep up with the rate of transpiration, loss of turgor occurs, and the stomata close. This immediately reduces the rate of transpiration (as well as of photosynthesis). If the loss of turgor extends to the rest of the leaf and stem, the plant **wilts**.

The volume of water lost in transpiration can be very high. It has been estimated that over the growing season, one acre of corn (maize) plants may transpire 400,000 gallons (1.5 million liters) of water. As liquid water, this would cover the field with a lake 15 inches (38 cm) deep. An acre of forest probably does even better.

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16.2D: Gas Exchange in Plants

In order to carry on photosynthesis, green plants need a supply of **carbon dioxide** and a means of disposing of **oxygen**. In order to carry on cellular respiration, plant cells need **oxygen** and a means of disposing of **carbon dioxide** (just as animal cells do). Unlike animals, plants have no specialized organs for gas exchange (with the few inevitable exceptions!). The are several reasons they can get along without them:

- Each part of the plant takes care of its own gas exchange needs. Although plants have an elaborate liquid transport system, it does not participate in gas transport.
- Roots, stems, and leaves respire at rates much lower than are characteristic of animals. Only during photosynthesis are large volumes of gases exchanged, and each leaf is well adapted to take care of its own needs.
- The distance that gases must diffuse in even a large plant is not great. Each living cell in the plant is located close to the surface. While obvious for leaves, it is also true for stems. The only **living** cells in the stem are organized in thin layers just beneath the bark. The cells in the interior are dead and serve only to provide mechanical support.
- Most of the living cells in a plant have at least part of their surface exposed to air. The loose packing of parenchyma cells in leaves, stems, and roots provides an interconnecting system of air spaces. Gases diffuse through air several thousand times faster than through water. Once oxygen and carbon dioxide reach the network of intercellular air spaces (arrows), they diffuse rapidly through them.
- Oxygen and carbon dioxide also pass through the cell wall and plasma membrane of the cell by diffusion. The diffusion of carbon dioxide may be aided by aquaporin channels inserted in the plasma membrane.

Leaves

The exchange of oxygen and carbon dioxide in the leaf (as well as the loss of water vapor in transpiration) occurs through pores called **stomata** (singular = stoma).

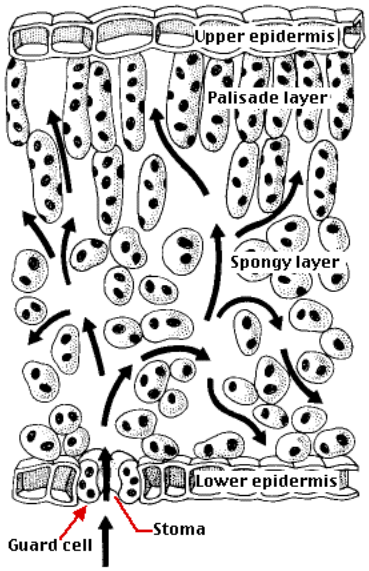


Figure 16.2.4.1 Stoma

Normally stomata open when the light strikes the leaf in the morning and close during the night. The immediate cause is a change in the turgor of the **guard cells**. The inner wall of each guard cell is thick and elastic. When turgor develops within the two guard cells flanking each stoma, the thin outer walls bulge out and force the inner walls into a crescent shape. This opens the stoma. When the guard cells lose turgor, the elastic inner walls regain their original shape and the stoma closes.

Time	Osmotic Pressure lb/in^2
7 A.M.	212

Time	Osmotic Pressure lb/in^2
11A.M.	456
5 P.M.	272
12 Midnight	191

The table shows the osmotic pressure measured at different times of day in typical guard cells. The osmotic pressure within the other cells of the lower epidermis remained constant at 150 lb/in^2 (~1000 kilopascal, kPa). When the osmotic pressure of the guard cells became greater than that of the surrounding cells, the stomata opened. In the evening, when the osmotic pressure of the guard cells dropped to nearly that of the surrounding cells, the stomata closed.

Opening stomata

The increase in osmotic pressure in the guard cells is caused by an uptake of **potassium** ions (K^+). The concentration of K^+ in open guard cells far exceeds that in the surrounding cells. This is how it accumulates:

- Blue light is absorbed by **phototropin** which activates a proton pump (an H^+-ATPase) in the plasma membrane of the guard cell.
- ATP, generated by the light reactions of photosynthesis, drives the pump.
- As protons (H^+) are pumped out of the cell, its interior becomes increasingly negative.
- This attracts additional potassium ions into the cell, raising its osmotic pressure.

Closing stomata

Although open stomata are essential for photosynthesis, they also expose the plant to the risk of losing water through transpiration. Some 90% of the water taken up by a plant is lost in transpiration. In angiosperms and gymnosperms (but not in ferns and lycopsids), Abscisic acid (ABA) is the hormone that triggers closing of the stomata when soil water is insufficient to keep up with transpiration (which often occurs around mid-day).

The mechanism:

- ABA binds to receptors at the surface of the plasma membrane of the guard cells.
- The receptors activate several interconnecting pathways which converge to produce
  - a rise in pH in the cytosol
  - transfer of Ca^{2+} from the vacuole to the cytosol
- These changes stimulate the loss of negatively-charged ions (anions), especially NO_3^- and Cl^-, from the cell and also the loss of K^+ from the cell.
- The loss of these solutes in the cytosol reduces the osmotic pressure of the cell and thus turgor.
- The stomata close.

Open stomata also provide an opening through which bacteria can invade the interior of the leaf. However, guard cells have receptors that can detect the presence of molecules associated with bacteria called pathogen-associated molecular patterns (PAMPs). LPS and flagellin are examples. When the guard cells detect these PAMPs, ABA mediates closure of the stoma and thus close the door to bacterial entry.

This system of **innate immunity** resembles that found in animals.

Density of stomata

The density of stomata produced on growing leaves varies with such factors as the **temperature**, **humidity**, and **light intensity** around the plant. It also depends on the the concentration of **carbon dioxide** in the air around the leaves. The relationship is **inverse**; that is, as the concentration of CO_2 goes up, the number of stomata produced goes down, and vice versa. **Some**

evidence:

- Plants grown in an artificial atmosphere with a high level of CO_2 have fewer stomata than normal.
- Herbarium specimens reveal that the number of stomata in a given species has been declining over the last 200 years — the time of the industrial revolution and rising levels of CO_2 in the atmosphere.

These data can be quantified by determining the **stomatal index**: the ratio of the number of stomata in a given area divided by the total number of stomata and other epidermal cells in that same area.

Q&A

How does the plant determine how many stomata to produce?

It turns out that the mature leaves on the plant detect the conditions around them and send a signal (its nature still unknown - but see below*) that adjusts the number of stomata that will form on the developing leaves.

Two experiments (reported by Lake et al., in **Nature**, **411**:154, 10 May 2001):

- When the mature leaves of the plant (**Arabidopsis**) are encased in glass tubes filled with high levels (720 ppm) of CO_2, the developing leaves have fewer stomata than normal even though they are growing in normal air (360 ppm).
- Conversely, when the mature leaves are given normal air (360 ppm CO_2) while the shoot is exposed to high CO_2 (720 ppm), the new leaves develop with the normal stomatal index.

*One signal that increases stomatal density in 2-day-old Arabidopsis seedlings (a different experimental setup than the one above) is a 45-amino acid peptide called **stomagen** that is released by mesophyll cells and induces the formation of stomata in the epidermis above.

Stomata reveal past carbon dioxide levels

Because CO_2 levels and stomatal index are inversely related, could fossil leaves tell us about past levels of CO_2 in the atmosphere? Yes. As reported by Gregory Retallack (in **Nature**, **411**:287, 17 May 2001), his study of the fossil leaves of the ginkgo and its relatives shows:

- their stomatal indices were **high** late in the Permian period (275–290 million years ago) and again in the Pleistocene epoch (1–8 million years ago). Both these periods are known from geological evidence to have been times of **low** levels of atmospheric carbon dioxide and ice ages (with glaciers).
- Conversely, stomatal indices were **low** during the Cretaceous period, a time of high CO_2 levels and warm climate.

These studies also lend support to the importance of carbon dioxide as a greenhouse gas playing an important role in global warming.

Roots and Stems

Woody stems and mature roots are sheathed in layers of dead cork cells impregnated with **suberin** — a waxy, waterproof (and airproof) substance. So cork is as impervious to oxygen and carbon dioxide as it is to water. However, the cork of both mature roots and woody stems is perforated by nonsuberized pores called **lenticels**. These enable oxygen to reach the intercellular spaces of the interior tissues and carbon dioxide to be released to the atmosphere.

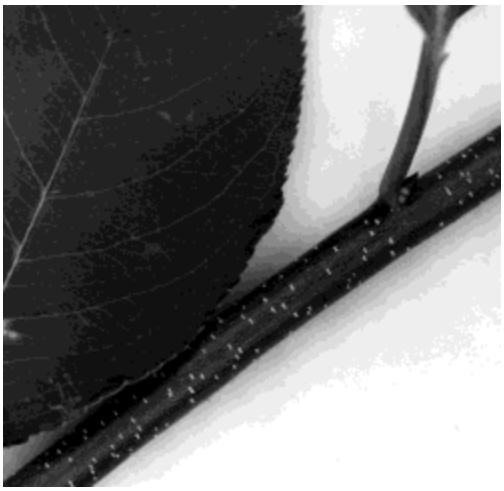


Figure 16.2.4.2 Lenticels. The photo shows the lenticels in the bark of a young stem.

In many annual plants, the stems are green and almost as important for photosynthesis as the leaves. These stems use stomata rather than lenticels for gas exchange.

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16.2E: Photorespiration and C4 Plants

All plants carry on photosynthesis by adding carbon dioxide (CO_2) to a phosphorylated 5-carbon sugar called **ribulose bisphosphate**. This reaction is catalyzed by the enzyme **ribulose bisphosphate carboxylase oxygenase** (**RUBISCO**). The resulting 6-carbon compound breaks down into two molecules of **3-phosphoglyceric acid** (**PGA**). These **3-carbon** molecules serve as the starting material for the synthesis of glucose and other food molecules. The process is called the Calvin cycle and the pathway is called the **C3** pathway.

Photorespiration

As its name suggests, **RUBISCO** catalyzes two different reactions:

- adding CO_2 to ribulose bisphosphate — the **carboxylase** activity
- adding O_2 to ribulose bisphosphate — the **oxygenase** activity

Which one predominates depends on the relative concentrations of O_2 and CO_2 with

- high CO_2, low O_2 favoring the carboxylase action
- high O_2, low CO_2 favoring the oxygenase action

The light reactions of photosynthesis liberate oxygen and more oxygen dissolves in the cytosol of the cell at higher temperatures. Therefore, high light intensities and high temperatures (above ~ 30°C) favor the second reaction.

The uptake of O_2 by RUBISCO forms the 3-carbon molecule 3-phosphoglyceric acid, just as in the Calvin cycle, and the 2-carbon molecule glycolate. The glycolate enters peroxisomes where it uses O_2 to form intermediates that enter mitochondria where they are broken down to CO_2. So this process **uses** O_2 and **liberates** CO_2 as cellular respiration does which is why it is called **photorespiration**. It undoes the good anabolic work of photosynthesis, reducing the net productivity of the plant. For this reason, much effort so far largely unsuccessful has gone into attempts to alter crop plants so that they carry on less photorespiration. The problem may solve itself. If atmospheric CO_2 concentrations continue to rise, perhaps this will enhance the net productivity of the world's crops by reducing losses to photorespiration.

C4 Plants

Over 8,000 species of angiosperms have developed adaptations which minimize the losses to photorespiration. They all use a supplementary method of CO_2 uptake which forms a 4-carbon molecule instead of the two 3-carbon molecules of the Calvin cycle. Hence these plants are called **C4** plants. (Plants that have only the Calvin cycle are thus **C3** plants). Some C_4 plants - called CAM plants - separate their C_3 and C_4 cycles by **time**, while other C_4 plants have structural changes in their leaf anatomy so that their C_4 and C_3 pathways are separated in different parts of the leaf with RUBISCO sequestered where the CO_2 level is high; the O_2 level low.

After entering through stomata, CO_2 diffuses into a **mesophyll cell**. Being close to the leaf surface, these cells are exposed to high levels of O_2, but they have no RUBISCO so cannot start photorespiration (nor the dark reactions of the Calvin cycle).

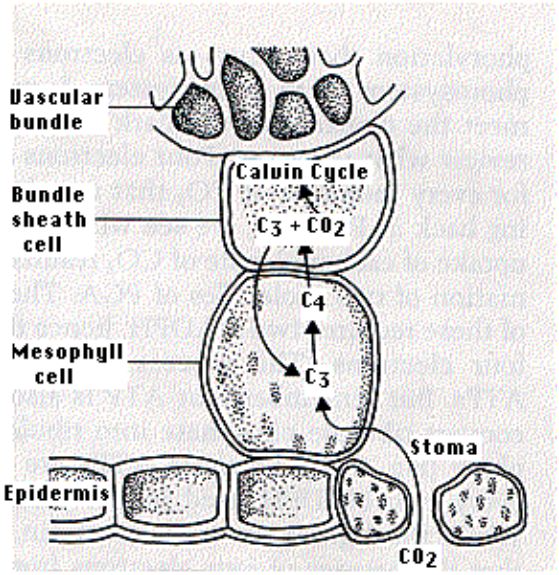


Figure 16.2.5.1 C4 Anatomy

Instead the CO_2 is inserted into a **3-carbon** compound (C_3) called **phosphoenolpyruvic acid** (**PEP**) forming the **4-carbon** compound **oxaloacetic acid** (**C_4**). Oxaloacetic acid is converted into malic acid or aspartic acid (both have 4 carbons), which is transported (by plasmodesmata) into a **bundle sheath cell**. Bundle sheath cells are deep in the leaf so atmospheric oxygen cannot diffuse easily to them and often have thylakoids with reduced photosystem II complexes (the one that produces O_2). Both of these features keep oxygen levels low in Bundle sheath cells, which is where the 4-carbon compound is broken down into **carbon dioxide**, which enters the Calvin cycle to form sugars and starch, and **pyruvic acid** (**C_3**), which is transported back to a mesophyll cell where it is converted back into **PEP**.

These C_4 plants are well adapted to (and likely to be found in) habitats with high daytime temperatures and intense sunlight. Some examples crabgrass, corn (maize), sugarcane, and sorghum. Although only ~3% of the angiosperms, C_4 plants are responsible for ~25% of all the photosynthesis on land.

4 cells in C_3 plants

The ability to use the C_4 pathway has evolved repeatedly in different families of angiosperms - a remarkable example of convergent evolution. Perhaps the potential is in all angiosperms.

A report in the 24 January 2002 issue of **Nature** (by Julian M. Hibbard and W. Paul Quick) describes the discovery that tobacco, a C_3 plant, has cells capable of fixing carbon dioxide by the C_4 path. These cells are clustered around the veins (containing xylem and phloem) of the stems and also in the petioles of the leaves. In this location, they are far removed from the stomata that could provide atmospheric CO_2. Instead, they get their CO_2 and/or the 4-carbon malic acid in the sap that has been brought up in the xylem from the roots.

If this turns out to be true of many C_3 plants, it would explain why it has been so easy for C_4 plants to evolve from C_3 ancestors.

CAM Plants

CAM plants are also C_4 plants (CAM stands for **c**rassulacean **a**cid **m**etabolism because it was first studied in members of the plant family *Crassulaceae*.). However, instead of segregating the C_4 and C_3 pathways in different parts of the leaf, CAM plants separate them in **time** instead (Table 1).

Table 1

Night	Morning
• CAM plants take in CO_2 through their open stomata (they tend to have reduced numbers of them). • The CO_2 joins with PEP to form the **4**-carbon oxaloacetic acid. • This is converted to **4**-carbon **malic acid** that accumulates during the night in the central vacuole of the cells.	• The stomata close (thus conserving moisture as well as reducing the inward diffusion of oxygen). • The accumulated malic acid leaves the vacuole and is broken down to release CO_2. • The CO_2 is taken up into the Calvin (C_3) cycle.

These adaptations also enable their owners to thrive in conditions of high daytime temperatures, intense sunlight, and low soil moisture. Some examples of CAM plants include cacti, Bryophyllum, the pineapple and all epiphytic bromeliads, sedums, and the "ice plant" that grows in sandy parts of the scrub forest biome.



Figure 1: Cultivated cacti in the Singapore Botanic Gardens. (CC SA-Y 3.0; Calvin Teo).

C_4 Diatoms

On 26 October 2000, **Nature** reported the discovery of both the C_3 and C_4 pathways in a marine diatom. In this **unicellular** organism, the two paths are kept separate by having the C_4 path in the cytosol, and the C_3 path confined to the chloroplast. The presence of a C_4 pathway probably reflects the frequent low concentrations of CO_2 in ocean waters.

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16.2F: Tropisms

This page explains tropisms in plants, which are growth movements influenced by external stimuli. There are positive (toward the stimulus) and negative (away from the stimulus) tropisms, with key types being phototropism (light) and gravitropism (gravity). Stems grow towards light (positively phototropic) and away from gravity (negatively gravitropic), while roots do the opposite.

A tropism is a **growth movement** whose direction is determined by the direction from which the stimulus strikes the plant.

- Positive = the plant, or a part of it, grows in the direction from which the stimulus originates.
- Negative = growth away from the stimulus.

Plants respond to:

- Light = **phototropism**
  - Stems are positively phototropic.
  - Roots are negatively phototropic.
- Gravity = **gravitropism**
  - Stems are negatively gravitropic while
  - roots are positively gravitropic.

The adaptive value of these tropisms is clear.

- Roots growing down and/or away from light are more likely to find the soil, water, and minerals they need.
- Stems growing up and toward the light will be able to expose their leaves so that photosynthesis can occur.

The Mechanism of Phototropism

The Darwin Experiments

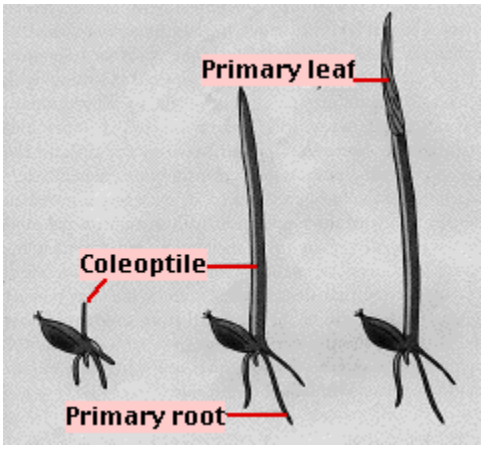


Figure 16.2.6.2 Darwins experiment

If they placed an opaque cover over the tip, phototropism failed to occur even though the rest of the coleoptile was illuminated from one side. However, when they buried the plant in fine black sand so that only its tip was exposed, there was no interference with the tropism — the buried coleoptile bent in the direction of the light.

From these experiments, it seemed clear that

- the stimulus (light) was detected at one location (the tip)
- the response (bending) was carried out at another (the region of elongation)

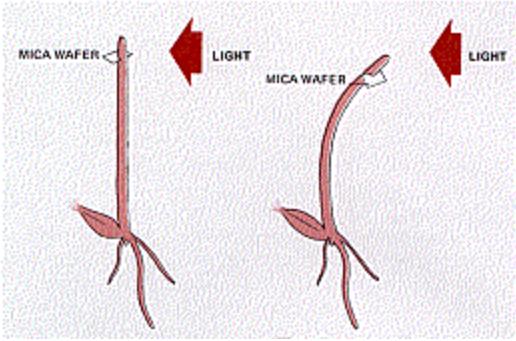


Figure 16.2.6.3 Boysen - Jensen experiment

The Danish plant physiologist Boysen-Jensen showed (in 1913) that the signal was a chemical passing down from the tip of the coleoptile.

He

- cut off the tip of the coleoptile
- covered the stump with a layer of gelatin
- replaced the tip.

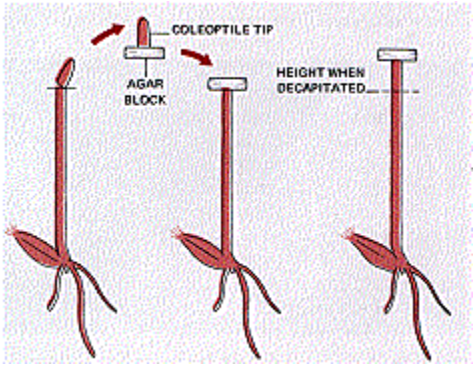


Figure 16.2.6.9 Roots vs Shoots

The graph (based on the work of K. V. Thimann) shows the effect of auxin concentration on root and stem growth. The difference between the behavior of roots and stems lies in the difference in the sensitivity of their cells to auxin. Auxin concentrations high enough to stimulate stem growth **inhibit** root growth.

Possible Mechanism of Gravitropism in Roots

When a root is placed on its side,

- Statoliths (organelles containing starch grains) settle by gravity to the bottom of cells in the **root tip**.
- This causes PIN proteins to redistribute to the underside of the cell where they pump **auxin** out of the cell.
- The auxin then accumulates along the **under** side of the root.
- This INHIBITS root cell elongation (see graph).
- So the cells at the **top** surface of the root elongate, causing the root to grow **down**.

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16.3: REPRODUCTION IN PLANTS



- Topic hierarchy

16.3A: Alternation of Generations in Plants

16.3B: Moss Life Cycle

16.3C: Fern Life Cycle

16.3D: Angiosperm Life Cycle

16.3E: Asexual Reproduction in Plants

16.3E: Self-incompatibility - How Plants Avoid Inbreeding

16.3F: Transgenic Plants

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16.3A: Alternation of Generations in Plants

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16.3B: Moss Life Cycle

Mosses and liverworts are traditionally classified together in the Division Bryophyta on the basis of their sharing a similar life cycle (alternation of generations), similar reproductive organs (antheridia and archegonia), and a lack of vascular tissue (xylem and phloem).



Figure 16.3.2.1: A thallose liverwort, Lunularia cruciata. Imaged used with permission (Public Domain; Velela).

Some 23,000 species of living mosses and liverworts have been identified. These are small, fairly simple, plants usually found in moist locations. Liverworts have a thin, leathery body that grows flat on moist soil or, in some cases, the surface of still water. The above photo is of a common liverwort, *Ricciocarpus natans*. Mosses have an erect shoot bearing tiny leaflike structures arranged in spirals. Neither mosses nor liverworts have any woody tissue so they never grow very large. They have neither xylem nor phloem for the transport of water and food through the plant.

The Gametophyte Generation

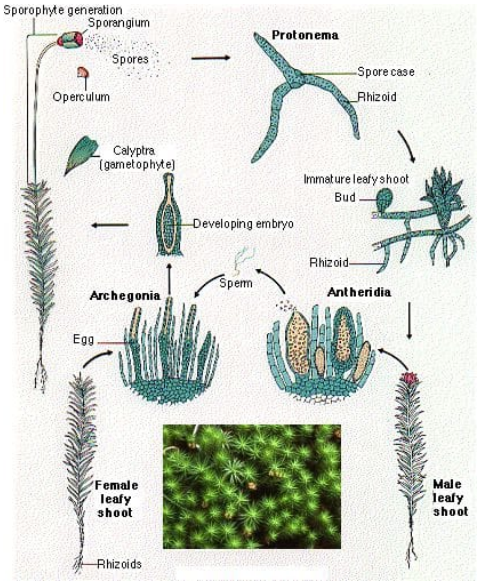


Fig.16.3.2.2 Moss life cycle

The leafy shoot of mosses is haploid and thus part of the gametophyte generation.

In the common haircap moss, *Polytrichum commune* (shown here), there are three kinds of shoots:

- **Female**, which develop **archegonia** at their tip.
  - A single **egg** forms in each archegonium.
- **Male**, which develop **antheridia** at their tip.
  - Multiple swimming **sperm** form in each antheridium.
- **Sterile**, which do not form sex organs.

In early spring, raindrops splash sperm from male to female plants. These swim down the canal in the archegonium to the chamber containing the egg. The resulting zygote begins the sporophyte generation.

The Sporophyte Generation



Figure 16.3.2.3 Moss sporophyte

Mitosis of the zygote produces an embryo that grows into the mature sporophyte generation. It consists of:

- A foot, which absorbs water, minerals, and food from the parent gametophyte
- A stalk, at the tip of which is formed a **sporangium** (the brownish objects in the photo).

The sporangium is

- filled with **spore mother cells**
- sealed by an **operculum**
- covered with a **calyptra**. The calyptra develops from the wall of the old archegonium and so is actually a part of the gametophyte generation. It is responsible for the common name ("haircap moss") of this species.

During the summer, each spore mother cell undergoes meiosis, producing four haploid spores - the start of the new gametophyte generation. Late in the summer, the calyptra and operculum become detached from the sporangium allowing the spores to be released. These tiny spores are dispersed so effectively by the wind that many mosses are worldwide in their distribution. If a spore reaches a suitable habitat, it germinates to form a filament of cells called a **protonema**. Soon buds appear and develop into the mature leafy shoots.

Thus the gametophyte generation is responsible for sexual reproduction. The sporophyte generation is responsible for dispersal.

Evolutionary Position of the Bryophytes

Evidence from the chloroplast genome

The chloroplasts of mosses and liverworts, like those of all photosynthetic eukaryotes, contain multiple copies of a small genome: circular DNA molecules encoding some - but not all of the genes needed for their own replication and photosynthesis. Chloroplast genomes have been sequenced from representatives of most of the plant groups. Although they all contain the same genes, they fall into two distinct groups with respect to the organization of their genes.

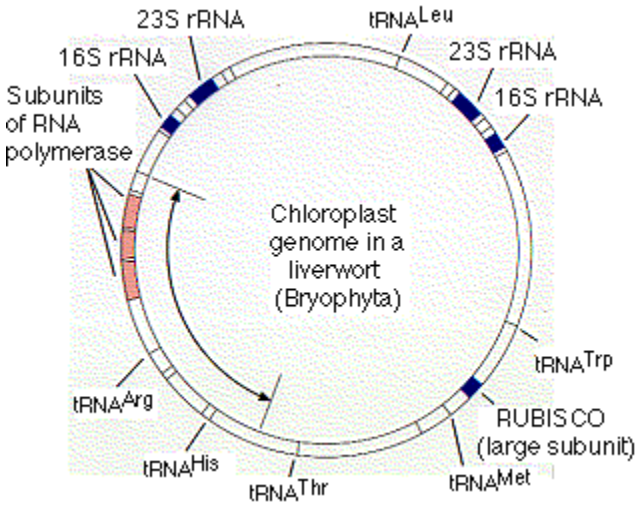


Figure 16.3.2.4 Chloroplast gene

- One group is illustrated by the figure, which shows the organization of the genome in ***Marchantia polymorpha***, a liverwort. Between the two arrowheads is a stretch of some 30,000 base pairs. The order of the genes in this region is also found in **mosses** (supporting the idea that mosses and liverworts are, indeed, close relatives), and in the **lycopsids** — vascular plants (they have xylem and phloem) that have a fossil record going back over 400 million years.
- In **all the other groups of plants**, the same genes are present but in **inverted order**.

Evidence from the mitochondrial genome (mtDNA)

However, the mitochondrial DNA of plants suggests a different evolutionary scenario. The mtDNA of all plants including **mosses** and **lycopsids** but NOT **liverworts** (nor **green algae**) contain certain shared introns. This suggests that:

- mosses and liverworts are not close relatives
- liverworts and lycopsids are not close relatives
- liverworts may have been the first group of plants to evolve from algal ancestors

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16.3C: Fern Life Cycle

Ther are over 10,000 species of ferns. Most are found in the tropics where tree ferns with their above-ground stems may grow as high as 40 feet. In temperate regions, the stems of ferns called **rhizome** grow underground. The leaves called **fronds** grow up from the rhizome each spring.

Alternation of Generations

The Sporophyte Generation

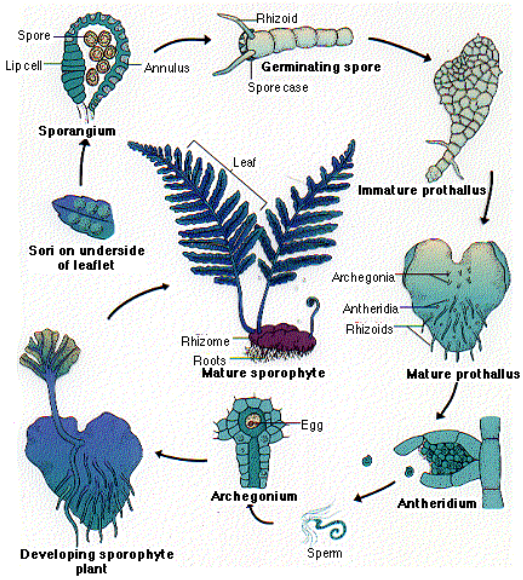


Figure 16.3.3.1 Fern life cycle

The plant we recognize as a fern is the diploid sporophyte generation. **Sori** form on the fronds. Each contains many **sporangia** mounted on stalks. Within each sporangium, the **spore mother cells** undergo meiosis producing four haploid spores each.

When the humidity drops,

- The thin-walled **lip cells** of each sporangium separate.
- The **annulus** slowly straightens out.
- Then the annulus snaps forward expelling the spores.



Figure 16.3.3.2 Sori on a fern leaflet

The photo shows the sori on the underside of the leaflets of *Polystichum acrostichoide*s, the Christmas fern.

The Gametophyte Generation

If a spore is blown to a suitable moist location, it germinates into a filament of cells. This may grow into a **prothallus** with

1. **rhizoids**, which absorb water and minerals from the soil,
2. **archegonia**, which produce a single egg (by mitosis) or
3. **antheridia**, which form **swimming sperm** (again, by mitosis) or
4. **both**.

Fertilization

If moisture is plentiful, the sperm swim to archegonia - usually on another prothallus because the two kinds of sex organs generally do not mature at the same time on a single prothallus. Another method for promoting cross-fertilization: The first spores to germinate develop into prothallia with archegonia. These prothallia secrete a gibberellin into their surroundings. This is absorbed by younger prothallia and causes them to produce antheridia exclusively. Fertilization restores the diploid number and begins a new sporophyte generation. The embryo sporophyte develops a **foot** that penetrates the tissue of the prothallus and enables the sporophyte to secure nourishment until it becomes self-sufficient. Although it is tiny, the haploid fern prothallus is a fully-independent, autotrophic plant.

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16.3D: Angiosperm Life Cycle

Angiosperms are the flowering plants (today the most abundant and diverse plants on earth). Most are terrestrial and all lack locomotion. This poses several problems. Gametes are delicate single cells. For two plants to cross fertilize, there must be a mechanism for the two gametes to reach each other safely. Moreover, there must also be a mechanism to disperse their offspring far enough away from the parent so that they do not have to compete with the parent for light, water, and soil minerals. The functions of the **flower** solve both of these problems.

The Flower and Its Pollination

In angiosperms, meiosis in the sporophyte generation produces two kinds of spores: (1) **microspores** which develop in the **microsporangium** and will germinate and develop into the male gametophyte generation and (2) **megaspores** that develop in the **megasporangium** will develop into the female gametophyte generation. Both types of sporangia are formed in flowers. In most angiosperms, the flowers are **perfect**: each has both microsporangia and megasporangia, although some angiosperms are **imperfect**, having either microsporangia or megasporangia but not both.

- **Monoecious** plants have both types of imperfect flower on the same plant.
- **Dioecious** plants have imperfect flowers on separate plants; that is, some plants are male, some female. Examples include willows, poplars, and the date palm. Most dioecious plants use an X-Y system of of sex determination like that in mammals. However, a few species use an X-to-autosome ratio system like that of *Drosophila*, and a very few use a ZW system like that of birds and lepidoptera.

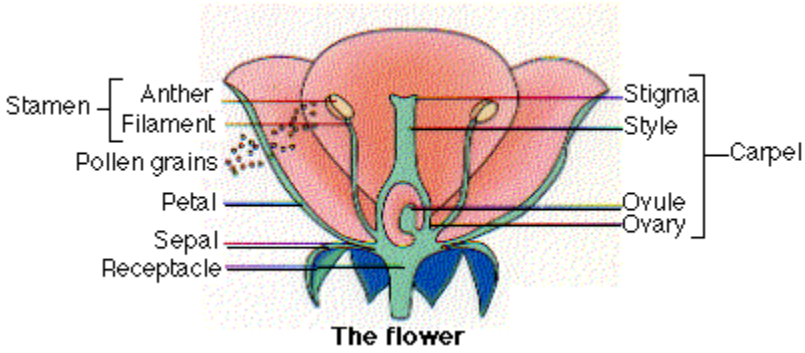


Figure 16.3.4.1 Flower

Flowers develop from flower buds. Each bud contains 4 concentric whorls of tissue. From the outer to the inner, these develop into

- a whorl of **sepals** (collectively called the **calyx**)
- a whorl of **petals** (collectively called the **corolla**)
- **stamens** in which the microsporangia form
- **carpels** in which the megasporangia form.

Stamens

Each stamen consists of a lobed **anther**, containing the microsporangia and supported by a thin **filament.** Meiosis of the diploid microspore mother cells in the anther produces four haploid **microspores**. Each of these develops into a **pollen grain** consisting of **a larger vegetative cell** (also called the tube cell) inside of which is a a smaller **germ cell** (also called the generative cell). At some point, depending on the species, the germ cell divides by mitosis to produce 2 **sperm cells**.

Carpels

Carpels consist of a **stigma**, usually mounted at the tip of a **style** with an **ovary** at the base. Often the entire whorl of carpels is

fused into a single **pistil**. The megasporangia, called **ovules**, develop within the ovary. Meiosis of the megaspore mother cell in each ovule produces 4 haploid cells, a large **megaspore** and 3 small cells that disintegrate.

Development of the megaspore

The nucleus of the megaspore undergoes three successive mitotic divisions. The 8 nuclei that result are distributed and partitioned off by cell walls to form the **embryo sac**. This is the **mature female gametophyte generation**. The **egg cell** will start the new sporophyte generation if it is fertilized. It is flanked by 2 synergid cells. In several (perhaps all) angiosperms, they secrete an attractant that guides the pollen tube through the **micropyle** into the embryo sac. The large **central cell**, which in most angiosperms contains 2 **polar nuclei**, will after its fertilization develop into the **endosperm** of the seed. It also contains 3 antipodal cells.

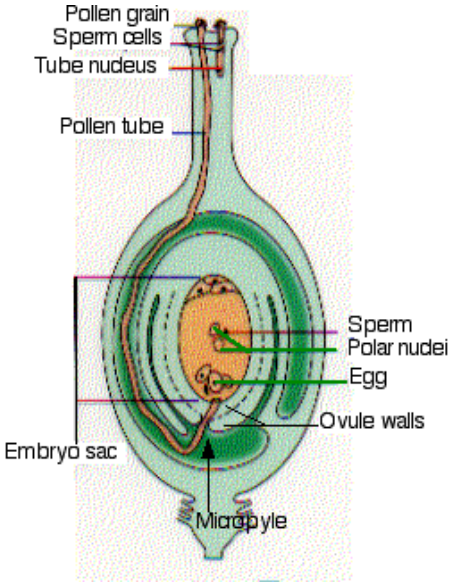


Figure 16.3.4.2 Embryo sac

Pollination

When a pollen grain reaches the stigma, it germinates into a **pollen tube**. If it hasn't done so already, the germ cell divides by mitosis forming 2 **sperm cells**. These, along with the tube nucleus (also known as the vegetative nucleus), migrate down the pollen tube as it grows through the style, the micropyle, and into the ovule chamber.

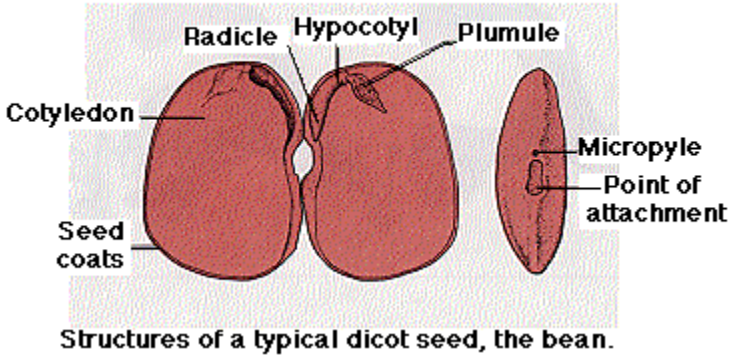
In Arabidopsis the pollen tube follows a gradient of increasing concentration of a small defensin-like protein secreted by the synergids. The pollen tube with its contents makes up the **mature male gametophyte generation**.

Double fertilization

The pollen tube enters the ovule through the micropyle and ruptures. One sperm cell fuses with the egg forming the diploid **zygote**. The other sperm cell fuses with the polar nuclei forming the **endosperm nucleus**. Most angiosperms have two polar nuclei so the endosperm is **triploid** (3n). The tube nucleus disintegrates.

Most angiosperms have mechanisms by which they avoid self-fertilization.

Seeds

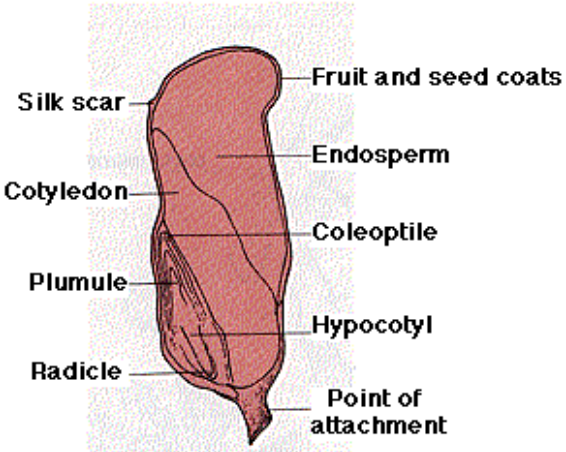


Structures of a typical dicot seed, the bean.

Figure 16.3.4.3 Bean seed

After double fertilization, **each ovule develops into a seed**, which consists of

- a **plumule**, made up of two embryonic leaves, which will become the first true leaves of the seedling, and a terminal (apical) bud. The terminal bud contains the meristem at which later growth of the stem takes place.
- One or two **cotyledons** which store food that will be used by the germinating seedling. Angiosperms that produce seeds with two cotyledons are called **dicots**. Examples include beans, squashes, Arabidopsis. Angiosperms whose seeds contain only a single cotyledon are **monocots**. Examples include corn and other grasses.
- The **hypocotyl** and **radicle**, which will grow into the part of the stem below the first node ("hypocotyl" = below the cotyledons) and primary root respectively. The development of each of the parts of the plant embryo depends on gradients of the plant hormone, auxin.
- In addition to the embryo plant (derived from the zygote), each seed is covered with protective seed coats derived from the walls of the ovule.



Structures of a corn kernel. Because its outer covering is derived from the ovary wall of the flower, the corn kernel is actually a fruit with a single seed inside.

Figure 16.3.4.4 Corn kernel

The food in the cotyledons is derived from the endosperm which, in turn, received it from the parent sporophyte. In many angiosperms (e.g., beans), when the seeds are mature, the endosperm has been totally consumed and its food transferred to the cotyledons. In others (some dicots and all monocots), the endosperm persists in the mature seed.

The seed is thus a dormant embryo sporophyte with stored food and protective coats. Its two functions are

- dispersal of the species to new locations (aided in angiosperms by the **fruit**)
- survival of the species during unfavorable climatic periods (e.g., winter). "Annual" plants (e.g., beans, cereal grains, many

weeds) can survive freezing only as seeds. When the parents die in the fall, the seeds remain alive — though dormant— over the winter. When conditions are once more favorable, **germination** occurs and a new generation of plants develops.

Fruits

Fruits are a development of the **ovary wall** and sometimes other flower parts as well. As seeds mature, they release the hormone auxin, which stimulates the wall of the ovary to develop into the fruit. In fact, commercial fruit growers may stimulate fruit development in unpollinated flowers by applying synthetic auxin to the flower.

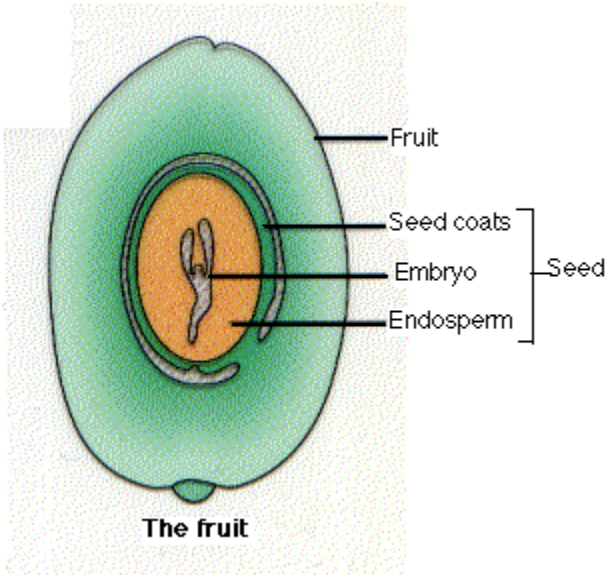


Figure 16.3.4.5 Fruit

Fruits promote the dispersal of their content of seeds in a variety of ways.

- Wind. The maple "key" and dandelion parachute are examples.
- Water. Many aquatic angiosperms and shore dwellers (e.g., the coconut palm) have floating fruits that are carried by water currents to new locations.
- Hitchhikers. The cocklebur and sticktights achieve dispersal of their seeds by sticking to the coat (or clothing) of a passing animal.
- Edible fruits. Nuts and berries entice animals to eat them. Buried and forgotten (nuts) or passing through their g.i. tract unharmed (berries), the seeds may end up some distance away from the parent plant.
- Mechanical. Some fruits, as they dry, open explosively expelling their seeds. The pods of many legumes (e.g., wisteria) do this.

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16.3E: Asexual Reproduction in Plants

Asexual reproduction is the formation of new individuals from the cell(s) of a single parent. It is very common in plants, less so in animals.

Asexual Reproduction in Plants

All plant organs have been used for asexual reproduction, but stems are the most common.

Stems

In some species, stems arch over and take root at their tips, forming new plants.



Figure 16.3.5.1 Strawberry stolon

The horizontal above-ground stems (called **stolons**) of the strawberry (shown here) produce new daughter plants at alternate nodes.

Underground stems such as rhizomes, bulbs, corms and tubers are used for asexual reproduction as well as for food storage.

Irises and day lilies, for example, spread rapidly by the growth of their rhizomes.

Leaves



Figure 16.3.5.2 Leaves of Bryophyllum

This photo shows the leaves of the common ornamental plant Bryophyllum (also called Kalanchoë) . Mitosis at meristems along the leaf margins produce tiny plantlets that fall off and can take up an independent existence.

Roots

Some plants use their roots for asexual reproduction. The dandelion is a common example. Trees, such as the poplar or aspen, send up new stems from their roots. In time, an entire grove of trees may form - all part of a **clone** of the original tree.

Plant Propagation

Commercially-important plants are often deliberately propagated by asexual means in order to keep particularly desirable traits (e.g., flower color, flavor, resistance to disease). Cuttings may be taken from the parent and rooted.

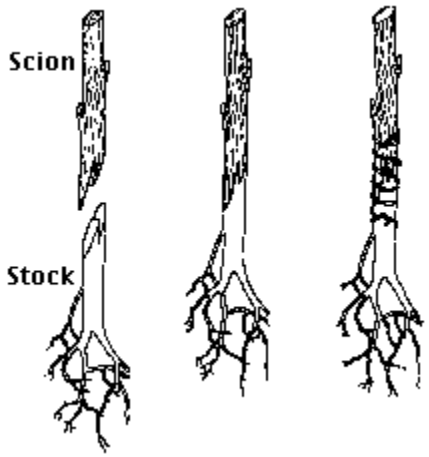


Figure 16.3.5.3 Grafting

Grafting is widely used to propagate a desired variety of shrub or tree. All apple varieties, for example, are propagated this way.

Apple **seeds** are planted only for the root and stem system that grows from them. After a year's growth, most of the stem is removed and a twig (**scion**) taken from a mature plant of the desired variety is inserted in a notch in the cut stump (the **stock**). So long the cambiums of scion and stock are united and precautions are taken to prevent infection and drying out, the scion will grow. It will get all its water and minerals from the root system of the stock. However, the fruit that it will eventually produce with be identical (assuming that it is raised under similar environmental conditions) to the fruit of the tree from which the scion was taken.

Apomixis

Citrus trees and many other species of angiosperms use their seeds as a method of asexual reproduction; a process called **apomixis**.

- In one form, the egg is formed with **2n** chromosomes and develops without ever being fertilized.
- In another version, the cells of the ovule (**2n**) develop into an embryo instead of - or in addition to - the fertilized egg.

Hybridization between different species often yields infertile offspring. But in plants, this does not necessarily doom the offspring. Many such hybrids use apomixis to propagate themselves.

The many races of Kentucky bluegrass growing in lawns across North America and the many races of blackberries are two examples of sterile hybrids that propagate successfully by apomixis.

Recently, an example of apomixis in gymnosperms was discovered (see Pichot, C., et al, in the 5 July 2001 issue of **Nature**). In a rare cypress, the pollen grains are diploid, not haploid, and can develop into an embryo when they land on either the female cones of their own species (rare) or those of a much more common species of cypress.

Is this **paternal apomixis** in a surrogate mother a desperate attempt to avoid extinction?

Breeding apomictic crop plants

Many valuable crop plants (e.g., corn) cannot be propagated by asexual methods like grafting.

Agricultural scientists would dearly love to convert these plants to apomixis: making embryos that are genetic clones of themselves rather than the product of sexual reproduction with its inevitable gene reshuffling. After 20 years of work, an apomictic corn (maize) has been produced, but it does not yet produce enough viable kernels to be useful commercially.

Asexual Reproduction in Animals

Budding

Here, offspring develop as a growth on the body of the parent. In some species, e.g., jellyfishes and many echinoderms, the buds break away and take up an independent existence. In others, e.g., corals, the buds remain attached to the parent and the process results in **colonies** of animals. Budding is also common among parasitic animals, e.g., tapeworms.

Fragmentation

As certain tiny worms grow to full size, they spontaneously break up into 8 or 9 pieces. Each of these fragments develops into a mature worm, and the process is repeated.

Parthenogenesis

In parthenogenesis ("virgin birth"), the females produce eggs, but these develop into young without ever being fertilized. Parthenogenesis occurs in some fishes, several kinds of insects, and a few species of frogs and lizards. It does not normally occur in mammals because of their imprinted genes. However, using special manipulations to circumvent imprinting, laboratory mice have been produced by parthenogenesis.

In a few nonmammalian species it is the only method of reproduction, but more commonly animals turn to parthenogenesis only under certain circumstances. Examples:

- Aphids use parthenogenesis in the spring when they find themselves with ample food. In this species, reproduction by parthenogenesis is more rapid than sexual reproduction, and the use of this mode of asexual reproduction permits the animals to quickly exploit the available resources.
- Female Komodo dragons (the largest lizard) can produce offspring by parthenogenesis when no male is available for sexual reproduction. Their offspring are homozygous at every locus including having identical sex chromosomes. Thus the females produce all males because, unlike mammals, females are the heterogametic sex (ZW) while males are homogametic (ZZ).

Parthenogenesis is forced on some species of wasps when they become infected with bacteria (in the genus **Wolbachia**). Wolbachia can pass to a new generation through eggs, but not through sperm, so it is advantageous to the bacterium for females to be made rather that males.

In these wasps (as in honeybees),

- fertilized eggs (diploid) become females
- unfertilized (haploid) eggs become males

However, in Wolbachia-infected females, all their eggs undergo endoreplication producing diploid eggs that develop into females without fertilization; that is, by parthenogenesis.

Treating the wasps with an antibiotic kills off the bacteria and "cures" the parthenogenesis!

Apis mellifera capensis

Occasionally worker honeybees develop ovaries and lay unfertilized eggs. Usually these are haploid, as you would expect, and develop into males. However, workers of the subspecies ***Apis mellifera capensis*** (the Cape honeybee) can lay unfertilized

diploid eggs that develop into females (who continue the practice). The eggs are produced by meiosis, but then the polar body nucleus fuses with the egg nucleus restoring diploidy (2n). (The phenomenon is called **automictic thelytoky**.)

Why Choose Asexual Reproduction?

Perhaps the better question is: **Why not?**

After all, asexual reproduction would seem a more efficient way to reproduce. Sexual reproduction requires males but they themselves do not produce offspring.

Two general explanations for the overwhelming prevalence of sexually-reproducing species over asexual ones are:

- Perhaps sexual reproduction has kept in style because it provides a mechanism to weed out (through the recombination process of meiosis) harmful mutations that arise in the population reducing its fitness. Asexual reproduction leads to these mutations becoming homozygous and thus fully exposed to the pressures of natural selection.
- Perhaps it is the ability to adapt quickly to a changing environment that has caused sex to remain the method of choice for most living things.

Purging Harmful Mutations

Most mutations are harmful - changing a functional allele to a less or nonfunctional one. An asexual population tends to be genetically static. Mutant alleles appear but remain forever associated with the particular alleles present in the rest of that genome. Even a beneficial mutation will be doomed to extinction if trapped along with genes that reduce the fitness of that population.

But with the **genetic recombination** provided by sex, new alleles can be shuffled into different combinations with all the other alleles available to the genome of that species. A beneficial mutation that first appears alongside harmful alleles can, with recombination, soon find itself in more fit genomes that will enable it to spread through a sexual population.

Evidence (from Paland and Lynch in the 17 February 2006 issue of **Science**):

Some strains of the water flea ***Daphnia pulex*** (a tiny crustacean) reproduce sexually, others asexually. The asexual strains accumulate deleterious mutations in their mitochondrial genes four times as fast as the sexual strains.

Evidence (from Goddard *et al*. in the 31 March 2005 issue of **Nature**):

Budding yeast missing two genes essential for meiosis adapt less rapidly to growth under harsh conditions than an otherwise identical strain that can undergo genetic recombination. Under good conditions, both strains grow equally well.

Evidence (from Rice and Chippindale in the 19 October 2001 issue of **Science**):

Using experimental Drosophila populations, they found that a beneficial mutation introduced into chromosomes that **can** recombine did - over time increase in frequency more rapidly than the same mutation introduced into chromosomes that could **not** recombine.

So sex provides a mechanism for testing **new combinations of alleles** for their possible usefulness to the phenotype:

- deleterious alleles weeded out by natural selection
- useful ones retained by natural selection

Some organisms may still gain the benefits of genetic recombination while avoiding sex. Many mycorrhizal fungi use asexual reproduction only. However, at least two species have been shown to have multiple - similar - copies of the same gene; that is, are polyploid. Perhaps recombination between these (during mitosis?) enables these organisms to avoid the hazards of accumulating deleterious mutations. (See the paper by Pawlowska and Taylor in the 19 Feb 2004 issue of **Nature**.)

But there are many examples of populations that thrive without sex, at least while they live in a **stable environment**.

Rapid Adaptation to a Changing Environment

As we have seen (above), populations without sex are genetically static. They may be well-adapted to a given environment, but will be handicapped in evolving in response to changes in the environment. One of the most potent environmental forces acting on a species environment is its parasites.

The speed with which parasites like bacteria and viruses can change their virulence may provide the strongest need for their hosts to have the ability to make new gene combinations. So sex may be virtually universal because of the never-ending need to keep up with changes in parasites.

Evidence:

- Some parasites interfere with sexual reproduction in their host:
  - Wolbachia-induced parthenogenesis discussed above is an example.
  - Several types of fungi block wind pollination of their grass hosts forcing them to inbreed with its resulting genetic uniformity.
- There is some evidence that genetically uniform populations are at increased risk of devastating epidemics and population crashes.
- Flour beetles (*Tribolium castaneum*) parasitized by the microsporidium *Nosema whitei* increase the rate of recombination during meiosis.
- *Drosophila* females parasitized by bacteria produce more recombinant offspring than non-infected mothers do.

The idea that a constantly-changing environment, especially with respect to parasites, drives evolution is often called the **Red Queen** hypothesis. It comes from Lewis Carroll's book *Through the Looking Glass*, where the Red Queen says "Now here, you see, it takes all the running you can do to keep in the same place".

The possibilities outlined above are not mutually exclusive and a recent study [see Morran, L. T., *et al*., in *Nature*, **462**:350, 19 November 2009] suggests that both forces are at work in favoring sexual reproduction over its alternatives.

The organism for testing these theories was ***Caenorhabditis elegans***. While *C. elegans* does not reproduce asexually, most worms are hermaphrodites and usually reproduce by self-fertilization with each individual fertilizing its own eggs. This quickly results in its genes becoming homozygous and thus fully-exposed to natural selection just as they are in asexually-reproducing species.

Hermaphrodites have two X chromosomes and self-fertilization ("selfing") usually produces more of the same; that is, hermaphrodites produce more hermaphrodites. However, an occasional nondisjunction generates an embryo with a single X chromosome and this develops into a male. These males can mate with hermaphrodites (their sperm is preferred over the hermaphrodites own) and, in fact, such "outcrossing" produces a larger number of offspring. It also produces 50% hermaphrodites and 50% males.

Testing the role of outcrossing vs. self-fertilization in maintaining fitness in the face of an increased mutation rate.

These workers developed six strains of worms:

- two that could reproduce only by selfing
- two that could reproduce only by crossing a male with an hermaphrodite ("outcrossing")
- "wild-type" worms

All the strains were exposed to a chemical mutagen that increased the spontaneous mutation rate some fourfold.

The results: After 50 generations, the

- the strains of worms that could reproduce only by selfing suffered a serious decline in fitness
- the strains of worms that could reproduce only by outcrossing suffered no decline
- the wild-type worms with intermediate levels of outcrossing (20–30%) suffered only moderate declines in fitness.

Fitness was measured by placing the worms in a petri dish with a barrier that they had to cross to reach their food (*E. coli*).

The conclusion: the genetic recombination provided by outcrossing protected the worms from loss of fitness even in the face of

an increase in mutation rate.

Testing the role of outcrossing vs. self-fertilization in the speed of adaptation to a changed environment.

For these tests, one of each category of mating types was exposed over 40 generations to a pathogenic bacterium (*Serratia marcescens*) that killed most worms when eaten by them.

The results: After 40 generations,

- the strain of worms that could reproduce only by selfing were just as susceptible to the pathogen as they were at the start while
- the strain of worms that could reproduce only by outcrossing had evolved a high degree of resistance to the pathogen
- the wild-type worms only developed a modest increase in their resistance to the bacteria.

Since these studies were reported, the same team has expanded their experiments to examine the effects of evolution in the pathogen (*Serratia marcescens*), that is, to look for evidence of **coevolution** of host and parasite. (Reported by Morran, L. T., *et al*., in *Science*, **333**: 216, 8 July 2011.)

Over 30 generations of worms, they harvested and tested the bacteria recovered from the bodies of worms that had died within 24 hours of infection. They found that:

- Worms that can maintain genetic variability by outcrossing suffered substantially lower mortality from the coevolved parasite that did worms from the starting population (kept frozen until used).
- Worms that could only reproduce by selfing became so susceptible to the evolving strain of *Serratia marcescens* that they died out within 20 generations.
- Curiously, the selection pressure of the increasing virulence of *Serratia marcescens* caused wild-type worms to increase their rate of outcrossing from the normal 20–30% to over 80%. So one response to the pressure of this coevolving parasite was to promote sex in its host.

Reproduction in Rotifers

Rotifers are microscopic invertebrates. They are assigned a phylum of their own (not discussed elsewhere in these pages). The phylum includes:

- a class of ~1,500 species called monogonont rotifers (they have only a single gonad). The monogonont rotifers can choose either asexual or sexual reproduction as circumstances warrant.
- a class of ~350 species called bdelloid rotifers. The bdelloid rotifers are limited to asexual reproduction only. Even after years of study, neither males nor haploid eggs have ever been found in any members of this group. It looks as though they gave up sexual reproduction millions of years ago.

Laboratory studies show that monogonont rotifers favor asexual reproduction when they are living in a stable environment but shift to more sexual reproduction when placed in a varied or unfavorable environment. As they adapt to the new environment, they gradually switch back to asexual reproduction.

But how have the bdelloid rotifers that never engage in sexual reproduction managed to survive? How have they avoided the demands of the Red Queen; that is, avoided extinction at the hands of parasites?

One study (Wilson, C. G. and Sherman, P. W., **Science**, **327**:574, 29 January 2010) reveals a mechanism. These tiny animals can be completely desiccated (dried out) and remain in suspended animation for years. In the desiccated state, they can be blown vast distances (some species are worldwide in their distribution). Once deposited in a moist environment (a few drops of water are sufficient), they resume an active life. Wilson and Sherman have shown that the desiccation that is harmless to the rotifers is lethal to their fungal parasite. So once dried, they are not only cured of their parasite, but can then be blown to a spot where they can resume an active life with no parasites present.

Another way in which these rotifers can avoid the evolutionary dead-end expected of asexually-reproducing organisms has been revealed by DNA sequencing of their genome. It turns out that they can purge their genome of deleterious alleles by gene conversion (during mitosis).

But in any case despite its disadvantages sexual reproduction is here to stay

- reducing the effect of harmful mutations
- increasing the speed with which populations can adapt to changes in their environment

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16.3E: Self-incompatibility - How Plants Avoid Inbreeding

Evolution seems to favor (and be favored by) genetic variability. Genetic variability is promoted by **outbreeding -** sexual reproduction between genetically dissimilar parents. Just why sexual reproduction is so popular throughout the world of living things is still a hotly-debated question, but the fact remains. Plants, being anchored in position, have a special problem in this regard. Many employ the services of animals (e.g., insects, birds, bats) to transfer pollen from plant to plant. But if the flowers have both sex organs:what is to prevent the pollen from fertilizing its own eggs?

A variety of solutions have been tried in the plant kingdom. These include:

- Having **imperfect flowers**; that is, flowers that are either male or female.
  - **Dioecy.** The imperfect flowers are present on separate plants. Dioecy is the equivalent of the separate sexes of most animals. But it is rather rare. Some examples include poplars and hollies.
  - **Monoecy.** The imperfect flowers are present on the same plant. But if they mature at different times, self-fertilization is avoided. Corn (maize) is a common example.

But the vast majority of angiosperms have **perfect flowers**; that is containing both male and female sex organs. So how do they avoid self-fertilization?

- **Heteromorphic flowers**.

  The flowers are perfect but come in two structural types; for example

  - long stamens with a short style
  - short stamens with a long style

- **Homomorphic flowers**. All flowers have exactly the same structure. Avoidance of self-fertilization depends on genetic/biochemical mechanisms. There are two quite different types of self-incompatibility.
  - **Sporophytic self-incompatibility** (SSI)
  - **Gametophytic self-incompatibility** (GSI)

Sporophytic Self-Incompatibility (SSI)

This form of self-incompatibility has been studied intensively in members of the mustard family (**Brassica**), including turnips, rape, cabbage, broccoli, and cauliflower.

Figure 16.3.6.1 S Loci

In this system,

- Rejection of self pollen is controlled by the diploid genotype of the sporophyte generation.
- The control lies in the "**S-locus**", which is actually a cluster of three tightly-linked loci:
  - ***SLG*** (**S**-**L**ocus **G**lycoprotein) which encodes part of a receptor present in the cell wall of the **stigma**;
  - ***SRK*** (**S**-**R**eceptor **K**inase), which encodes the other part of the receptor. Kinases attach phosphate groups to other proteins. SRK is transmembrane protein embedded in the plasma membrane of the **stigma** cell.
  - ***SCR*** (**S**-locus **C**ysteine-**R**ich protein), which encodes a soluble ligand for the **same** receptor which is secreted by the pollen.
- Because the plants cannot fertilize themselves, they tend to be heterozygous; that is, carry a pair of different S loci (here designated S_1 and S_2).
- However, **dozens** of different S alleles may be present in the **population** of the species; that is; the S-locus in the species is extremely polymorphic (analogous to the major histocompatibility locus of vertebrates).
- The difference between the alleles is concentrated in certain "**hypervariable regions**" of the receptor (analogous to the hypervariable regions that provide the great binding diversity of **antibodies**

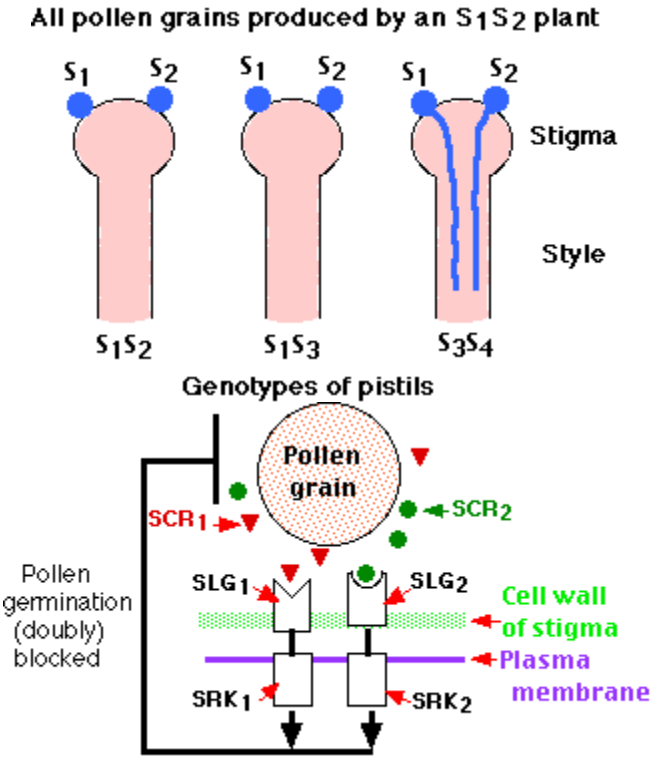


Figure 16.3.6.3 Pollen Stigma SSI

The rules:

- Pollen will not germinate on the stigma (diploid) of a flower that contains **either** of the two alleles in the sporophyte parent that produced the pollen.
- This holds true even though each pollen grain being haploid contains only one of the alleles.
- In the example shown here, the S_2 pollen, which was produced by a S_1S_2 parent, cannot germinate on an S_1S_3 stigma.

The explanation:

- The S_1S_2 pollen-producing sporophyte synthesizes both SCR_1 and SCR_2 for incorporation in (and later release from) **both** S_1 and S_2 pollen grains.
- If **either** SCR molecule can bind to **either** receptor on the pistil, the kinase triggers a series of events that lead to failure of the stigma to support germination of the pollen grain. Among these events is the ubiquination of proteins targeting them for destruction in proteasomes.
- If this path is not triggered (e.g., pollen from an S_1S_2 parent on an S_3S_4 stigma, the pollen germinates successfully.

Gametophytic Self-Incompatibility (GSI)

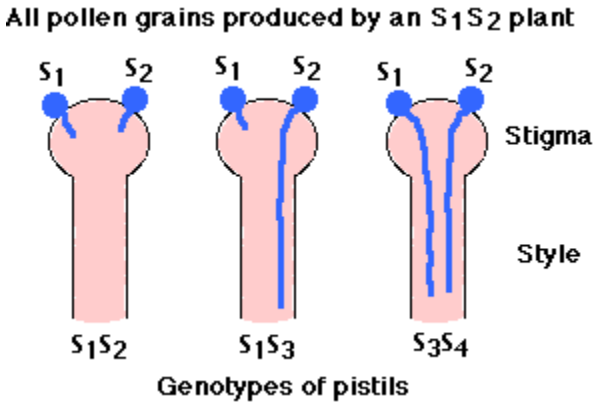


Figure 16.3.6.4 GSI

This form of self-incompatibility is more common than SSI but not so well understood. It occurs in nearly one-half of all the families of angiosperms, including

- the Solanaceae (potatoes, tomatoes [wild, not cultivated], and tobacco)
- petunias
- beets (*Beta vulgaris*)
- buttercups (Ranunculus)
- lilies
- roses
- many grasses

The rules:

- The S loci are (as in SSI plants) extremely polymorphic; that is, there is an abundance of multiple alleles in the population.
- Incompatibility is controlled by the single S allele in the haploid pollen grain.
- Thus a pollen grain will grow in any pistil that does not contain the same allele (so, as shown here and in contrast to what happens in SSI, S_2 pollen from an S_1S_2 parent will grow down an S_1S_3 style.

This appears to be the mechanism in the petunia:

- All pollen grains - incompatible as well as compatible - germinate forming pollen tubes that begin to grow down the style.
- However, growth of incompatible pollen tubes stops in the style while compatible tubes go on to fertilize the egg in the ovary.
- The block within incompatible pollen tubes is created by an S-locus-encoded **ribonuclease** (S-RNase), which is synthesized within the **style,** enters the pollen tube and destroys its RNA molecules halting pollen tube growth.
- The RNase molecules contain a **hypervariable region**, each encoded by a different allele, which establishes each S specificity (S_1, S_2, S_3, etc.).
- The pollen tube expresses a protein designated SLF that binds S-RNase. SLF also exists in different S specificities (S_1, S_2, S_3, etc.).
- In **compatible** ("nonself") tubes, the SCF triggers the degradation (in proteasomes) of the S-RNase thus permitting RNAs in the pollen tube to survive and growth to continue.
- In **incompatible** ("self") tubes the interaction of, for example, the S_1 SCF with the S_1 S-RNase blocks its degradation so the

RNAs of the pollen tube are destroyed and growth is halted.

An entirely-different mechanism of gametophytic self-incompatibility is found in poppies (*Papaver rhoeas*).

Switching from Cross-Pollination to Self-Pollination

A substantial minority of angiosperms have abandoned cross-pollination for self-pollination. For example, while its wild relatives continue to be cross-pollinated, the domestic tomato is not. Two steps are needed for this change:

1. abandoning its mechanism of self-incompatibility
2. changes in flower structure to reduce the chance that pollinators will transfer pollen from another plant to its stigma.

Unlike its wild relatives, the stigma of the domestic tomato does not protrude beyond the anthers. Of the several genes involved in this change, the most important one is *Style2.1*. The mutation in *Style2.1* responsible for the change in phenotype in our cultivated tomatoes is found in the promoter region - the protein-encoding portion of the gene is exactly the same as in wild tomatoes.

Here, again, is evidence that much of the diversity of life arises not from mutations in the protein-coding portion of the genes that we share but **mutations in their regulatory regions** (promoters and enhancers).

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16.3F: Transgenic Plants

Progress is being made on several fronts to introduce new traits into plants using recombinant DNA technology. The genetic manipulation of plants has been going on since the dawn of agriculture, but until recently this has required the slow and tedious process of cross-breeding varieties. Genetic engineering promises to speed the process and broaden the scope of what can be done.

Making transgenic plants

There are several methods for introducing genes into plants, including infecting plant cells with plasmids as vectors carrying the desired gene or shooting microscopic pellets containing the gene directly into the cell. In contrast to animals, there is no real distinction between somatic cells and germline cells. Somatic tissues of plants, e.g., root cells grown in culture, can be transformed in the laboratory with the desired gene or grown into mature plants with flowers. If all goes well, the transgene will be incorporated into the pollen and eggs and passed on to the next generation. In this respect, it is easier to produce transgenic plants than transgenic animals.

Some Achievements

Improved Nutritional Quality

Milled rice is the staple food for a large fraction of the world's human population. Milling rice removes the husk and any beta-carotene it contained. Beta-carotene is a precursor to vitamin A, so it is not surprising that vitamin A deficiency is widespread, especially in the countries of Southeast Asia. The synthesis of beta-carotene requires a number of enzyme-catalyzed steps. In January 2000, a group of European researchers reported that they had succeeded in incorporating **three transgenes** into rice that enabled the plants to manufacture beta-carotene in their endosperm.

Insect Resistance

Bacillus thuringiensis is a bacterium that is pathogenic for a number of insect pests. Its lethal effect is mediated by a protein toxin it produces. Through recombinant DNA methods, the toxin gene can be introduced directly into the genome of the plant where it is expressed and provides protection against insect pests of the plant.

Disease Resistance

Genes that provide resistance against plant viruses have been successfully introduced into such crop plants as tobacco, tomatoes, and potatoes.



Figure 16.3.7.1 Tomatoes

Tomato plants infected with **tobacco mosaic virus** (which attacks tomato plants as well as tobacco). The plants in the back row carry an introduced gene conferring resistance to the virus. The resistant plants produced three times as much fruit as the sensitive plants (front row) and the same as control plants.

Herbicide Resistance

Questions have been raised about the safety - both to humans and to the environment - of some of the broad-leaved weed killers like 2,4-D. Alternatives are available, but they may damage the crop as well as the weeds growing in it. However, genes for resistance to some of the newer herbicides have been introduced into some crop plants and enable them to thrive even when exposed to the weed killer.

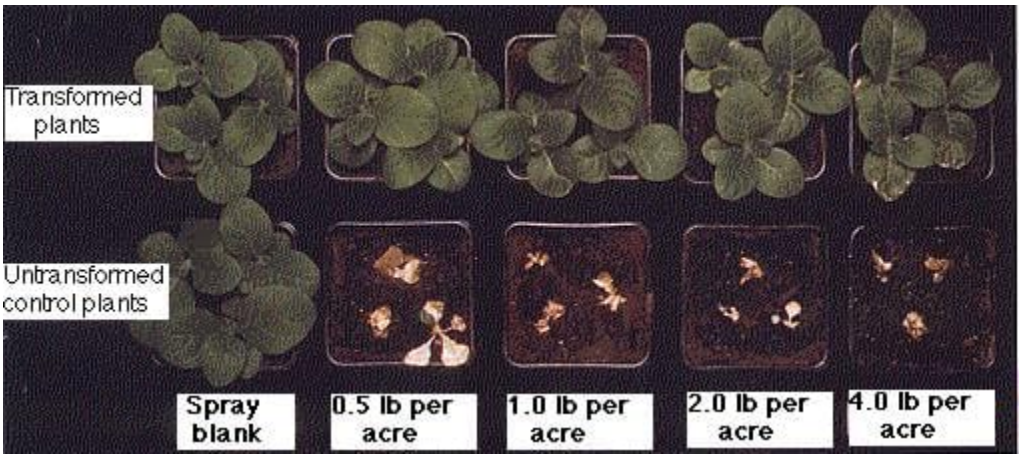


Figure 16.3.7.2 Effect of bromoxynil on tobacco courtesy of Calgene, Davis, CA

Effect of the herbicide bromoxynil on tobacco plants transformed with a bacterial gene whose product breaks down bromoxynil (top row) and control plants (bottom row). "Spray blank" plants were treated with the same spray mixture as the others except the bromoxynil was left out.

Salt Tolerance

A large fraction of the world's irrigated crop land is so laden with salt that it cannot be used to grow most important crops. However, researchers at the University of California Davis campus have created transgenic tomatoes that grow well in saline soils. The transgene was a highly-expressed sodium/proton antiport pump that sequestered excess sodium in the vacuole of leaf cells. There was no sodium buildup in the fruit.

"Terminator" Genes

This term is used (by opponents of the practice) for transgenes introduced into crop plants to make them produce sterile seeds (and thus force the farmer to buy fresh seeds for the following season rather than saving seeds from the current crop). The process involves introducing three transgenes into the plant:

- A gene encoding a **toxin** which is lethal to developing seeds but not to mature seeds or the plant. This gene is normally inactive because of a stretch of DNA inserted between it and its promoter.
- A gene encoding a **recombinase** — an enzyme that can remove the spacer in the toxin gene thus allowing to be expressed.
- A **repressor** gene whose protein product binds to the promoter of the recombinase thus keeping it inactive.

How they work

When the seeds are soaked (before their sale) in a solution of tetracycline

- Synthesis of the repressor is blocked.
- The recombinase gene becomes active.
- The spacer is removed from the toxin gene and it can now be turned on.

Because the toxin does not harm the growing plant - only its developing seeds - the crop can be grown normally except that its seeds are sterile.

The use of terminator genes has created much controversy:

- Farmers - especially those in developing countries - want to be able to save some seed from their crop to plant the next season.
- Seed companies want to be able to keep selling seed.

Transgenes Encoding Antisense RNA

These are discussed in a separate page. Link to it

Biopharmaceuticals

The genes for proteins to be javascript:void('Remove anchor')used in human (and animal) medicine can be inserted into plants and expressed by them.

Advantages:

- Glycoproteins can be made (bacteria like E. coli cannot do this).
- Virtually unlimited amounts can be grown in the field rather than in expensive fermentation tanks.
- It avoids the danger from using mammalian cells and tissue culture medium that might be contaminated with infectious agents.
- Purification is often easier.

Corn is the most popular plant for these purposes, but tobacco, tomatoes, potatoes, rice and carrot cells grown in tissue culture are also being used. Some of the proteins that have been produced by transgenic crop plants:

- human growth hormone with the gene inserted into the chloroplast DNA of tobacco plants
- humanized antibodies against such infectious agents as
    - HIV
    - respiratory syncytial virus (RSV)
    - sperm (a possible contraceptive)
    - herpes simplex virus, HSV, the cause of "cold sores"
    - Ebola virus, the cause of the often-fatal Ebola hemorrhagic fever
- protein antigens to be used in vaccines
    - An example: patient-specific antilymphoma (a cancer) vaccines. B-cell lymphomas are clones of malignant B cells

expressing on their surface a unique antibody molecule. Making tobacco plants transgenic for the RNA of the variable (unique) regions of this antibody enables them to produce the corresponding protein. This can then be incorporated into a vaccine in the hopes (early trials look promising) of boosting the patient's immune system - especially the cell-mediated branch - to combat the cancer.

- other useful proteins like lysozyme and trypsin
- However, as of April 2012, the only protein to receive approval for human use is glucocerebrosidase, an enzyme lacking in Gaucher's disease. It is synthesized by transgenic carrot cells grown in tissue culture.

Controversies

The introduction of transgenic plants into agriculture has been vigorously opposed by some. There are a number of issues that worry the opponents. One of them is the potential risk of transgenes in commercial crops endangering native or nontarget species.

Examples:

- A gene for herbicide resistance in, e.g. maize (corn), escaping into a weed species could make control of the weed far more difficult.
- The gene for Bt toxin expressed in pollen might endanger pollinators like honeybees.

To date, field studies on Bt cotton and maize show that the numbers of some nontarget insects are reduced somewhat but not as much as in fields treated with insecticides.

Another worry is the inadvertent mixing of transgenic crops with nontransgenic food crops. Although this has occurred periodically, there is absolutely no evidence of a threat to human health.

Despite the controversies, farmers around the world are embracing transgenic crops. Currently in the United States over 80% of the corn, soybeans, and cotton grown are genetically modified (GM) — principally to provide

- resistance to the herbicide glyphosate ("Roundup Ready®") thus making it practical to spray the crop with glyphosate to kill weeds without harming the crop
- resistance to insect attack (by expressing the toxin of Bacillus thuringiensis)

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16.4: PLANT DEVELOPMENT - FUNDAMENTALS

16.4A: Plant Growth
16.4B: Germination of Seeds
16.4C: Etiolation
16.4D: Flowering
16.4E: Photoperiodism and Phytochrome

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16.4A: Plant Growth

Growth in plants occurs chiefly at **meristems** where rapid **mitosis** provides new cells. As these cells differentiate, they provide new plant tissue.

Stem Growth

In **stems**, mitosis in the **apical meristem** of the shoot apex (also called the **terminal bud**) produces cells that enable the stem to grow longer and, periodically, cells that will give rise to leaves. The point on the stem where leaves develop is called a **node**. The region between a pair of adjacent nodes is called the **internode**. The internodes in the **terminal bud** are very short so that the developing leaves grow above the apical meristem that produced them and thus protect it. New meristems, the **lateral buds**, develop at the nodes, each just above the point where a leaf is attached. When the lateral buds develop, they produces new stem tissue, and thus **branches** are formed.

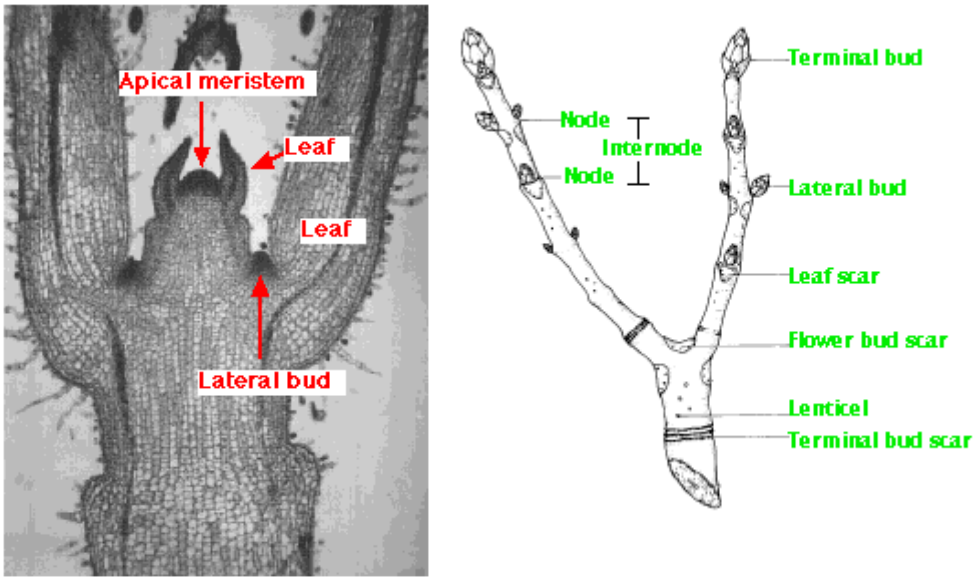


Figure 16.4.1.2 Horse chestnut

Under special circumstances (such as changes in photoperiod), the apical meristem is converted into a **flower bud**. This develops into a flower. The conversion of the apical meristem to a flower bud "uses up" the meristem so that no further growth of the stem can occur at that point. However, lateral buds behind the flower can develop into branches.

The drawing is of a typical woody dicot, the horse chestnut, as seen during the dormant season. The leaves have dropped off, leaving a **leaf scar**; the dots inside each leaf scar show where the vascular bundles (xylem and phloem) had entered the petiole of the leaf. A flower had been produced the season before, so that during the season just ended two branches had grown out on either side of the **flower bud scar**. **Lenticels** are openings that allow oxygen and carbon dioxide to diffuse between the living cells of the stem and the air.

The growth of roots in described in a separate page,

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16.4B: Germination of Seeds

Germination is the resumption of growth of the embryo plant inside the seed.

Requirements:

- proper **temperature**
- Water is always needed to allow vigorous metabolism to begin. It is also sometimes needed to leach away a germination inhibitor within the seed. This is especially common among desert annuals. The inhibitor is often abscisic acid (ABA).
- **oxygen**
- a preceding period of **dormancy** (often).

  The seeds of many temperate-climate angiosperms will germinate only after a prolonged period of cold. An inhibitor within the seed (probably abscisic acid - ABA) is gradually broken down at low temperatures until finally there is not enough to prevent germination when other conditions become favorable. This mechanism is of obvious survival value in preventing seeds from germinating during an unseasonably warm spell in the autumn or winter.

- Correct photoperiod (often).

Germination in a Dicot

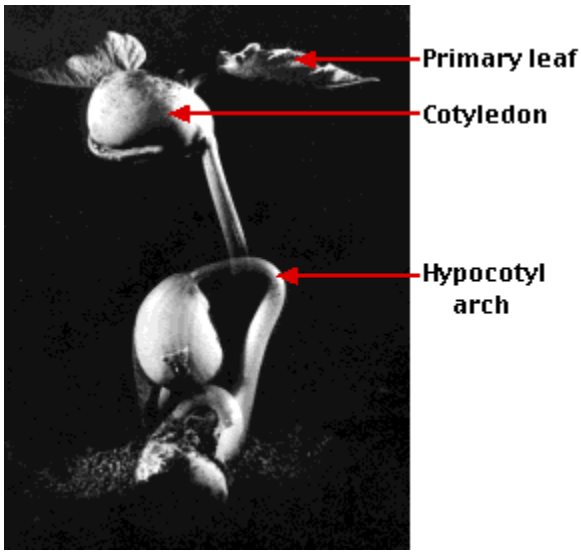


Figure 16.4.2.1 Germination of a bean seed

- The **primary root** emerges through the seed coats while the seed is still buried in the soil.
- The **hypocotyl** ("below the cotyledons") emerges from the seed coats and pushes its way up through the soil. It is bent in a hairpin shape - the **hypocotyl arch-** as it grows up. The **two cotyledons** protect the **plumule-** the **epicotyl** ("above the cotyledons") and first leaves - from mechanical damage.
- Once the hypocotyl arch emerges from the soil, it straightens out. This response is triggered by light. Both red light, absorbed by phytochrome and blue light, absorbed by **cryptochrome** can do the job.
- The cotyledons spread apart exposing the **epicotyl** with the apical meristem at its tip, and two **primary leaves**
- In many dicots, the cotyledons not only transfer their food stores to the developing plant but also turn green and make more food by photosynthesis until they drop off.

The above image (courtesy of the Pittsburgh Plate Glass Co.) is a time-lapse photograph showing three stages in the germination of a bean seed.

Germination in Monocots

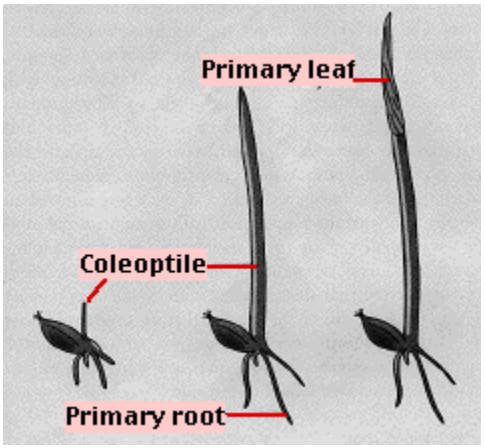


Figure 16.4.2.2 Coleoptile of an oat seed

When grass seeds like corn (maize) or oats (shown here) germinate,

- The **primary root** pierces the seed (and fruit) coverings and grows down.
- The **primary leaf** of the plant grows up. It is protected as it pushes up through the soil by the **coleoptile -** a hollow, cylindrical structure.
- Once the seedling has grown above the surface, the coleoptile stops growing and the primary leaf pierces it.

The coleoptile of grass (e.g., oat) seedlings has been a favorite experimental object for studing **phototropism**.

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16.4C: Etiolation

The stems of plants raised in the dark elongate much more rapidly than normal, a phenomenon called **etiolation**. It is a mechanism that increases the probability of the plant reaching the light.

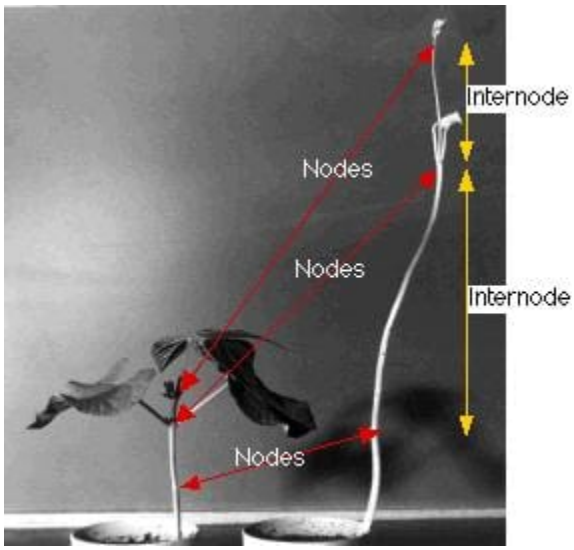


Figure 16.4.4.1 Etiolation

Once light shines on it,

- the cotyledons spread apart
- the primary leaves
  - grow to full size
  - turn green
- the plant begins to produce internodes of normal size.

The image shows seedlings of the common garden bean grown in the light (left) and in darkness (right). The pale color of the dark-grown plant is caused by the lack of chlorophyll. When the food reserves of its seed are used up, the seedling will die (unless placed in the light).

Each seedling has three nodes (the bottom ones where the cotyledons were attached), but the internodes are greatly elongated in the dark-grown seedling. This response is NOT dependent on photosynthesis, because light too dim to be useful in photosynthesis nevertheless halts etiolation. Exposure to dim red light (660 nm) or blue light can halt etiolation. The effect of blue light is mediated by cryptochrome. The red light effect is mediated by phytochrome.

How does phytochrome work?

The prevention of etiolation in Arabidopsis is mediated by **phytochrome B**.

- When sunlight (660 nm) converts P_R into P_{FR}, the P_{FR} moves from the cytoplasm into the nucleus.
- There it stimulates the activity of DELLA proteins.
- These bind to members of a group of helix-loop-helix proteins called **PIFs** ("phytochrome-interacting factors").
- PIFs, like many helix-loop-helix proteins, are transcription factors. They bind to and turn on promoters of genes that, among other effects, stimulate cell and thus stem elongation.
- However, when DELLA proteins bind PIFs, the PIFs are prevented from binding to the promoters of their target genes.
- The result: reduced cell elongation and so no etiolation.

Gibberellins are plant hormones that promote stem elongation thus mimicking the etiolation response. They do this by triggering

the degradation of DELLA proteins thus freeing PIFS to bind to the promoters of genes needed for cell elongation.

The Shade-Avoidance Response

Natural sunlight contains about the same amount of red (660 nm) as far-red (735 nm) light. However, chlorophyll absorbs 660 nm light strongly, so that the light that filters through a canopy of foliage is enriched in far-red light.

- This is absorbed by P_{FR} converting it into inactive P_R and relieving the inhibition of PIFs.
- The now-active PIFs turn on the genes needed for the synthesis of auxin.
- Auxin stimulates stem elongation.

The shade-avoidance response helps ensure that the plant reaches enough sunlight to be able to carry on photosynthesis.

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16.4D: Flowering

The flowering plants (angiosperms) go through a phase of **vegetative growth** producing more stems and leaves and a flowering phase where they produce the organs for sexual reproduction. In "**annuals**", like the snapdragon, the vegetative phase begins with germination of the seed. Flowering follows and ends with the senescence and death of the plant. In **biennials**, the vegetative phase takes up the first year; flowering followed by death occurs the second year. In **perennials**, flowering typically occurs year after year when conditions are appropriate.

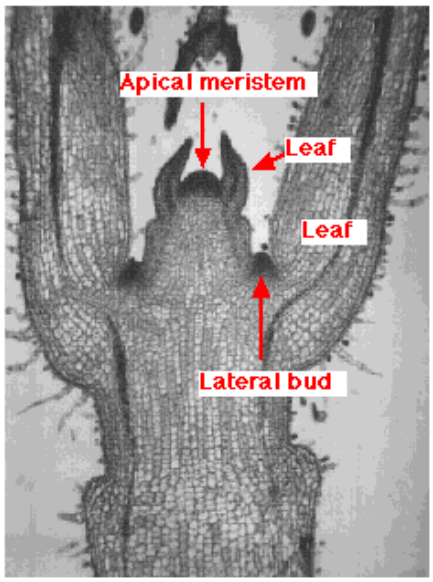


Figure 16.4.5.1 Meristem

Vegetative growth of the above-ground part of the plant — the **shoot** — occurs at the **apical meristem**. This is a mass of undifferentiated cells at the tip of the stem. Mitosis of these cells produces cells that differentiate to form more stem, leaves and **secondary meristems**. Also called **lateral buds**, these form in the axils of the leaves and will form branches.

The Signal to Flower

Flowering involves the conversion of the apical meristem into a **floral meristem**, from which all the parts of the flower will be produced. Signals that change the fate of the apical meristem include:

- maturity of the plant
- temperature
- the arrival of the plant hormone gibberellin
- for many plants, **photoperiod -** the relative length of day and night.

Temperature

Many annual plants (e.g., winter wheat) and biennial plants have their time of flowering delayed unless they have undergone a preceding period of wintertime cold. The change brought about by this prolonged exposure to the cold is called **vernalization**.

In the "model" plant *Arabidopsis thaliana*, vernalization works like this.

- A gene designated *Flowering Locus C* (*FLC*) encodes a transcription factor that blocks the expression of the genes needed for flowering.
- The level of FLC mRNA is high in the fall.
- But with the onset of cold temperatures, production of an antisense transcript of *FLC* (called *COOLAIR*) increasesas does, later, a sense transcript of part of the *FLC* gene.

- Both of these RNAs are non-coding; that is, they are not translated into protein.
- But they cooperate in suppressing the production of FLC mRNA and its translation into FLC protein.
- With the arrival of spring, there is no FLC protein remaining to suppress flowering so flowering can begin.

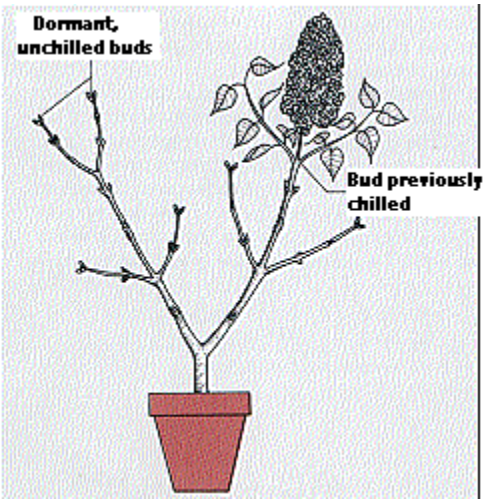


Figure 16.4.5.2 Lilac

The buds of many species of woody angiosperms found in temperate climates, such as apples and lilacs, also need a preceding period of cold weather before they can develop into flowers. So these plants cannot be grown successfully at lower latitudes because the winters never get cold enough (a few days at 0–10°C). This bud dormancy is localized. Prior chilling of one bud on a lilac stem enables it to flower while the other, nonchilled, buds on the stem remain dormant.

Photoperiod

Photoperiod is detected in the leaves. The cocklebur, drawn here, needs at least 8.5 hours of darkness in order to flower. Even if only a part of one leaf is exposed to the correct photoperiod, the entire plant will bloom (middle figure).

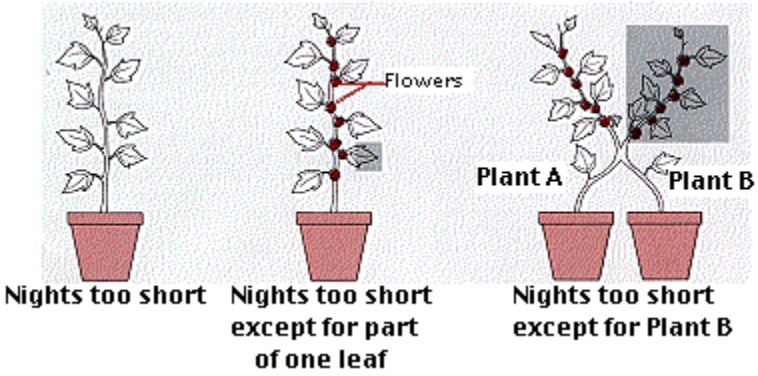


Figure 16.4.5.3 Photoperiod demonstration

The leaves produce a chemical signal called **florigen** that is transmitted to the apical meristems to start their conversion into floral meristems. The right-hand drawing shows that grafting a cocklebur (**B**) that receives the required period of darkness to one (**A**) that does not causes flowering in both. Evidently the florigen signal passes from **B** to **A** through their connected vascular systems.

The chemical nature of florigen has been sought for decades. The most recent evidence suggests that at least one component is the protein encoded by the gene *FLOWERING LOCUS T* (***FT***).

Converting the Apical Meristem to a Floral Meristem

In the nucleus of the meristem cells, the FT protein binds to the transcription factor **FD** and turns on the expression of genes

needed for flowering, e.g., ***APETALA1*** and ***LEAFY***.

Structure of the Flower

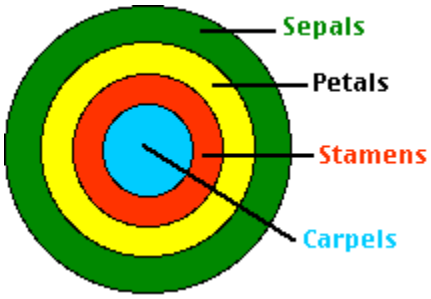


Figure 16.4.5.4 Floral meristem

The floral meristem differentiates into four concentric groups of cells that form the four parts of the flower.

1. The cells in whorl **1** develop into a whorl of **sepals**. These form at the lowest level. Collectively they make up the **calyx**.
2. Whorl **2** forms above the calyx, forming the **petals**. Collectively these make up the **corolla** of the flower (the part that most ornamentals are grown for).
3. Whorl **3** develops into the **stamens**, the **male** reproductive organs.
4. The innermost whorl, **4**, forms **carpels**, the female reproductive organs. Carpels often fuse to form a single structure, which some botanists call the **pistil**.

What triggers the various parts of the floral meristem to enter one or another of these four developmental pathways?

The ABC Model of Flower Development

Genetic analysis of mutants especially those found in the dicots ***Arabidopsis thaliana*** and in the snapdragon (***Antirrhinum***) support the ABC model of flowering. This model postulates a group of genes that encode the transcription factors needed to turn on the genes for sepal, petal, etc. development. The "master switches" fall into 3 groups: **A**, **B**, and **C**.

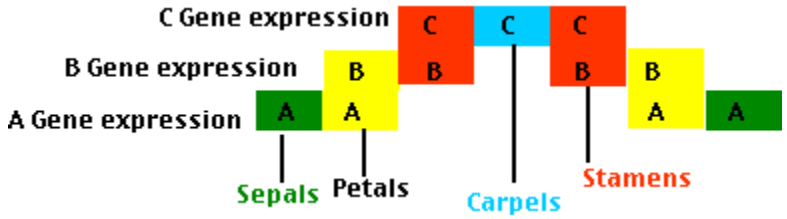


Figure 16.4.5.5 ABC Model

These are the rules:

- Cells in which only **A genes** are expressed develop into **sepals**. This occurs at the lowest level of the floral meristem.
- Cells in which both **A and B genes** are expressed develop into **petals**. This occurs at the next higher level.
- Expression of **B and C genes** turns on the developmental program to form **stamens**.
- Expression of **C genes** alone turns on the development of **carpels** in the innermost band of cells.

Examples of A, B and C group genes involved in flowering - these have been identified in ***Arabidopsis thaliana***

A group	***APETALA1*** (***AP1***) and ***APETALA2*** (***AP2***)
B group	***APETALA3*** (***AP3***) and ***PISTILLATA*** (***PI***)
C group	***AGAMOUS*** (***AG***)

The transcription factor **LEAFY** plays a major role in turning on the A, B, and C group genes in the appropriate locations.

- The LEAFY protein **alone** turns on ***AP1*** in whorls **1** and **2**.
- LEAFY plus a protein encoded by the gene ***UFO*** (for "unusual floral organs") turn on ***AP3*** in whorls **2** and **3**.
- LEAFY and a second, still unidentified, protein turn on ***AG*** in whorls **3** and **4**.

If LEAFY protein alone is sufficient to turn on *AP1*, why isn't *AP1* expressed in all four whorls?

The answer: **AGAMOUS** blocks the expression of ***AP1***, so any cell expressing AGAMOUS cannot express AP1. In fact, the antagonism is reciprocal: AP2 in whorls 1 and 2 (A group) inhibits AGAMOUS so the gene expression in whorls 3 and 4 remains distinct from that in whorls 1 and 2.

The proteins encoded by ***APETALA3*** and ***PISTILLATA*** (Group B) form a heterodimer that binds to

- the **APETALA1** protein to form **petals**
- the **AGAMOUS** protein to form **stamens**

Aided by a fourth transcription factor encoded by the gene *SEPALLATA3*, these quaternary complexes bind to specific sequences of DNA turning on the expression of the various genes needed to form whorls 2 and 3. Further research may reveal similar behavior for the other genes.

SEPALLATA3 (*SEP3*) is one of four *SEP* genes in Arabidopsis. If all but *SEP4* are inactivated, a flower with only sepals is formed (hence the name). If all four are inactivated, no flowers are formed at all.

So formation of a flower requires a **cascade of sequential gene activity** that gradually converts a mass of undifferentiated cells (the apical meristem) into the parts of a flower. The genes encode transcription factors that act as master switches, turning on (or off) downstream genes that ultimately make each part of the flower in its appropriate location. This same strategy of genetic control of developmental pathways is seen in animal development.

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16.4E: Photoperiodism and Phytochrome

Many angiosperms flower at about the same time every year. This occurs even though they may have started growing at different times. Their flowering is a response to the changing length of day and night as the season progresses. The phenomenon is called **photoperiodism**. It helps promote cross pollination.

In 1920 two employees of the U. S. Department of Agriculture, W. W. Garner and H. A. Allard, discovered a mutation in tobacco - a variety called Maryland Mammoth - that prevented the plant from flowering in the summer as normal tobacco plants do. Maryland Mammoth would not bloom until late December. Experimenting with artificial lighting in winter and artificial darkening in summer, they found that Maryland Mammoth was affected by photoperiod. Because it would flower only when exposed to short periods of light, they called it a **short-day** plant. Some other short-day plants are

- chrysanthemums (bloom in the fall)
- rice (*Oryza sativa*)
- poinsettias
- morning glory (*Pharbitis nil*)
- the cocklebur (Xanthium)

Some plants such as spinach, **Arabidopsis,** sugar beet and the radish flower only after exposure to long days and hence are called **long-day** plants. Still other plants, e.g. the tomato, are day neutral; that is, flowering is not regulated by photoperiod.

Photoperiodism also explains why some plant species can be grown only in a certain latitude. Spinach, a long-day plant, cannot flower in the tropics because the days never get long enough (14 hours). Ragweed, a short-day plant, fails to thrive in northern Maine because by the time the days become short enough to initiate flowering, a killing frost in apt to occur before reproduction and the formation of seeds is completed.

Photoperiodism in a Short-Day Plant

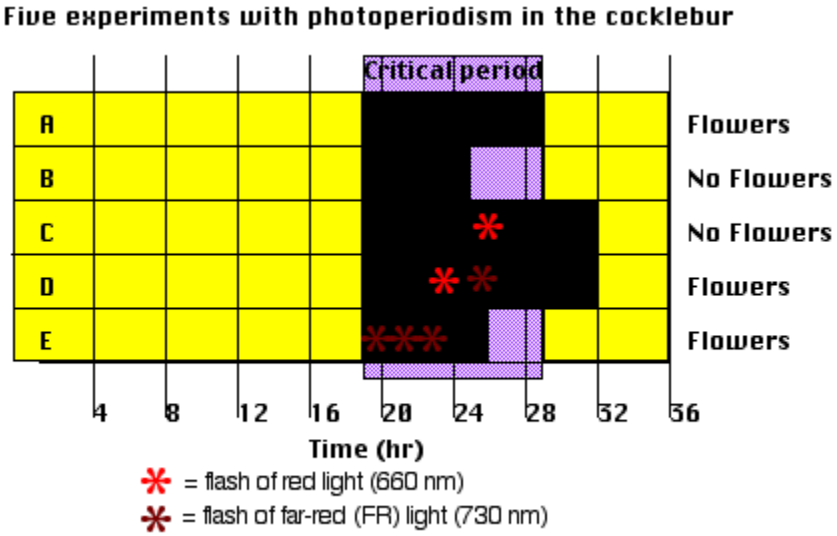


Figure 16.4.6.1 Cocklebur chart

Experiments with the cocklebur have shown that the term short-day is something of a misnomer; what the cocklebur needs is a sufficiently **long night**.

- Cockleburs (adapted to the latitude of Michigan) will flower only if they have been kept in the dark for at least 8.5 hours — the **critical period**. (**A** and **B**).
- Interruption of an otherwise long night by light — **red** (**660 nm**) rays are particularly effective — prevents flowering. (**C**) unless
- it is followed by irradiation with **far-red** (**730 nm**) light (**D**).

- An intense exposure to far-red light at the start of the night reduces the dark requirement by 2 hours (**E**).

These response are mediated by phytochrome.

Phytochrome

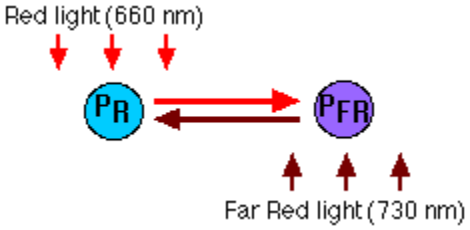


Figure 16.4.6.2 Pr_Pfr

- Phytochrome is a homodimer: two identical protein molecules each conjugated to a light-absorbing molecule (compare rhodopsin).
- Plants make 5 phytochromes: **PhyA**, **PhyB**, as well as C, D, and E.
- There is some redundancy in function of the different phytochromes, but there also seem to be functions that are unique to one or another. The phytochromes also differ in their absorption spectrum; that is, which wavelengths (e.g., red vs. far-red) they absorb best.
- Phytochromes exist in two interconvertible forms
- **P_R** because it absorbs red (R; **660 nm**) light
- **P_{FR}** because it absorbs far-red (FR; **730 nm**) light
- These are the relationships:
  - Absorption of red light by **P_R** converts it into **P_{FR}**.
  - Absorption of far-red light by **P_{FR}** converts it into **P_R**.
  - In the dark, **P_{FR}** spontaneously converts back to **P_R**.

The Hourglass Model

The behavior of phytochrome provided the first model - called the hourglass model - of the mechanism of photoperiodism in short-day plants.

- Sunlight is richer in red (660 nm) than far-red (730 nm) light, so at sundown all the phytochrome is P_{FR}.
- During the night, the P_{FR} converts back to P_R.
- The P_R form is needed for the release of the flowering signal.
- Therefore, the cocklebur needs 8.5 hours of darkness in which to
  - convert all the P_{FR} present at sundown into P_R
  - carry out the supplementary reactions leading to the release of the flowering signal ("florigen").
- If this process is interrupted by a flash of 660-nm light, the P_R is immediately reconverted to P_{FR} and the night's work is undone (**C**)
- A subsequent exposure to far-red (730 nm) light converts the pigment back to P_R and the steps leading to the release of "florigen" can be completed (**D**)
- Exposure to intense far-red light at the beginning of the night sets the clock ahead about 2 hours or so by eliminating the need for the spontaneous conversion of P_{FR} to P_R (**E**).

The Circadian Rhythm Model

Recent work mostly in the **long-day** plant, Arabidopsis - supports a different model of photoperiodism. This work suggests that the photoperiodic response is governed by the interaction of daylight with innate circadian rhythms of the plant.

- Virtually all eukaryotes have innate circadian rhythms.
- These are rhythms of biological activities that fluctuate over a period of approximately 24 hours (L. *circa* = about; *dies* = day) even under **constant environmental conditions** (e.g. continuous darkness). Under constant conditions, the cycles may drift

out of phase with the environment.
- However, when exposed to the environment (e.g., alternating day and night), the rhythms become **entrained**; that is, they now cycle in lockstep with the cycle of day and night with a period of exactly 24 hours.
- In Arabidopsis, the entrainment of the rhythms requires that light is detected by the
  - phytochromes (absorb red light)
  - cryptochromes (absorb blue light)

Long-Day Plants

Arabidopsis is a long-day plant and has provided many clues about the mechanism involved in this photoperiodic response. Any response to photoperiod requires a method of keeping time; that is, a clock. Plants, like so many other organisms, have an innate circadian rhythm that regulates the expression of many genes. Among these in Arabidopsis is *CONSTANS* (*CO*), a gene that encodes a zinc-finger transcription factor whose levels of mRNA rise and fall with a circadian rhythm.

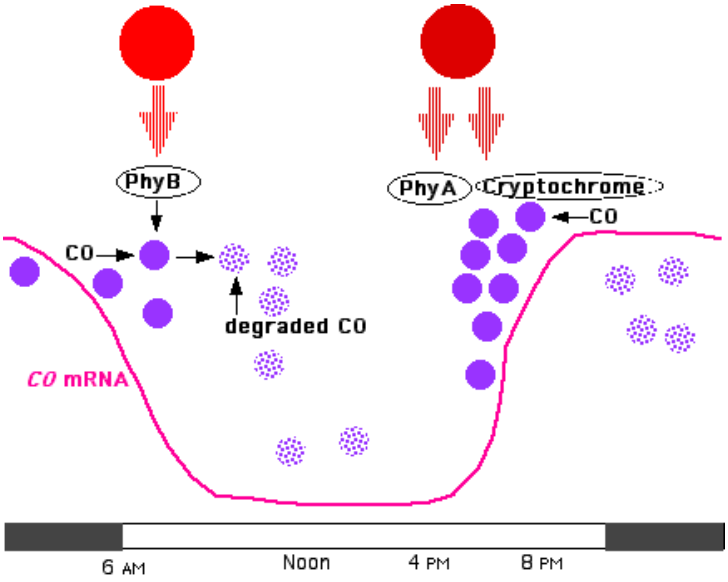


Figure 16.4.6.3 Co_mRNA

Translation of CONSTANS mRNA produces the transcription factor that turns on a number of genes, including ***FLOWERING LOCUS T*** (***FT***), a gene needed to start the conversion of apical buds into flower buds.

CONSTANS **messenger RNA** (mRNA) is abundant early in the morning, declines during the middle part of the day and then rises to another peak late in the afternoon.

However,

- the CONSTANS **protein** is quickly degraded (in proteasomes) during the morning and middle part of the day and also during the night.
- The degradation triggered by morning light (rich in 660 nm rays) is mediated by phytochrome B (PhyB).
- By late in the afternoon, if the day has been long enough,
  - transcription of the *CO* gene increases producing a rise in CO mRNA
  - translation of the CO mRNA produces more CO protein which is
  - no longer degraded.
- These effects are mediated by the absorption of
  - red (enriched in far-red) light by phytochrome A (PhyA)
  - blue light by cryptochrome.

Now with the CONSTANS protein accumulating, it is available to turn on the gene transcription (e.g., *FT*) needed for the induction of the flowering. In short days, with darkness falling before the rise in *CONSTANS* mRNA, there is not enough CONSTANS protein synthesized to induce flowering. So flowering in Arabidopsis seems to require the interaction of daylight perceived by phytochromes and cryptochromes with the intrinsic circadian rhythm of *CONSTANS* expression.

Short-Day Plants

The roles of circadian rhythms and light in short-day plants are not yet as well understood. Studies with rice, a short-day plant, suggests that the mechanism described for Arabidopsis may work there as well but with CONSTANS acting as a **suppressor** of *FLOWERING LOCUS T* and thus as an **inhibitor** of flowering under long days.

Trees

Photoperiodism not only controls flowering in some trees but also stops vegetative growth and promotes the setting of winter buds as the days grow shorter in the autumn. In aspens (*Populus* sp.) this control is mediated by CONSTANS and FLOWERING LOCUS T.

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16.5: PLANT DEVELOPMENT - HORMONES



- Topic hierarchy

16.5A: Abscisic acid (ABA)

16.5B: Auxin

16.5C: Cytokinins

16.5D: Ethylene

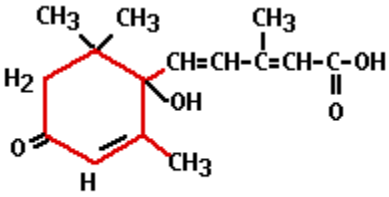
16.5E: Gibberellins

16.5F: Strigolactones

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16.5A: Abscisic acid (ABA)

Unlike animals, plants cannot flee from potentially harmful conditions like drought, freezing, exposure to salt water or salinated soil. They must adapt or die. The plant hormone abscisic acid (ABA) is the major player in mediating the adaptation of the plant to stress.



Abscisic acid (ABA)

Figure 16.4.3.1.1 ABA

Here are a few examples.

Closing of stomata

Some 90% of the water taken up by a plant is lost in transpiration. Most of this leaves the plant through the pores called stomata - in the leaf. Each stoma is flanked by a pair of **guard cells**. When the guard cells are turgid, the stoma is open. When turgor is lost, the stoma closes. In angiosperms and gymnosperms (but not in ferns and lycopsids), ABA is the hormone that triggers closing of the stomata when soil water is insufficient to keep up with transpiration.

The mechanism:

- ABA binds to receptors at the surface of the plasma membrane of the guard cells.
- The receptors activate several interconnecting pathways which converge to produce
  - a rise in pH in the cytosol
  - transfer of Ca^{2+} from the vacuole to the cytosol.
- These changes stimulate the loss of negatively-charged ions (anions), especially $NO3^{-}$ and Cl^{-}, from the cell and also the loss of K^{+} from the cell.
- The loss of these solutes in the cytosol reduces the osmotic pressure of the cell and thus turgor.
- The stomata close.

Protecting cells from dehydration

ABA signaling turns on the expression of genes encoding proteins that protect cells - in seeds as well as in vegetative tissues - from damage when they become dehydrated.

Root growth

ABA can stimulate root growth in plants that need to increase their ability to extract water from the soil.

Bud dormancy

ABA mediates the conversion of the apical meristem into a dormant bud. The newly developing leaves growing above the meristem become converted into stiff bud scales that wrap the meristem closely and will protect it from mechanical damage and drying out during the winter. ABA in the bud also acts to enforce dormancy so if an unseasonably warm spell occurs before winter is over, the buds will not sprout prematurely. Only after a prolonged period of cold or the lengthening days of spring

(photoperiodism) will bud dormancy be lifted.

Seed maturation and dormancy

Seeds are not only important agents of reproduction and dispersal, but they are also essential to the survival of annual and biennial plants. These angiosperms die after flowering and seed formation is complete. ABA is essential for seed maturation and also enforces a period of seed dormancy. As we saw for buds, it is important the seeds not germinate prematurely during unseasonably mild conditions prior to the onset of winter or a dry season. ABA in the seed enforces this dormancy. Not until the seed has been exposed to a prolonged cold spell and/or sufficient water to support germination is dormancy lifted.

Abscission

ABA also promotes abscission of leaves and fruits (in contrast to auxin, which inhibits abscission). It is, in fact, this action that gave rise to the name **abscisic** acid.

The dropping of leaves in the autumn is a vital response to the onset of winter when ground water is frozen - and thus cannot support transpiration - and snow load would threaten to break any branches still in leaf.

Most nondeciduous species in cold climates (e.g., pines) have "needles" for leaves. These are very narrow and have a heavy waterproof cuticle. The shape aids in shedding snow, and the cuticle cuts down on water loss.

Seedling growth

ABA inhibits stem elongation probably by its inhibitory effect on gibberellic acid.

Apical dominance

ABA - moving up from the roots to the stem - synergizes with auxin - moving down from the apical meristem to the stem - in suppressing the development of lateral buds. The result is inhibition of branching or apical dominance.

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16.5B: Auxin

Auxins are plant hormones. The most important auxin produced by plants is **indole-3-acetic acid** (**IAA**). It plays important roles in a number of plant activities, including:

- development of the embryo
- leaf formation
- phototropism
- gravitropism
- apical dominance
- fruit development
- abscission
- root initiation and development
- the shade-avoidance effect

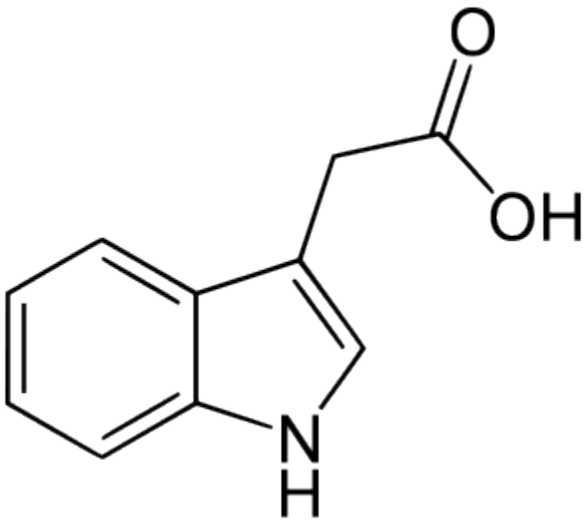


Figure 16.4.3.2.1: indole-3-acetic acid (IAA)

Embryonic Development

From the very first mitotic division of the zygote, gradients of auxin guide the patterning of the embryo into the parts that will become the organs of the plant:

- shoot apex,
- primary leaves,
- cotyledon(s),
- stem,
- root.

Leaf Formation

The formation of new leaves in the apical meristem is initiated by the accumulation of auxin. Already-developing leaves deplete the surrounding cells of auxin so that the new leaves do not form too close to them. In this way, the characteristic pattern of leaves in the plant is established. Auxin also controls the precise patterning of the epidermal cells of the developing leaf.

Phototropism

Plant **shoots** display **positive phototropism**: when illuminated from one direction, the shoot proceeds to grow in that direction. Proposed Mechanism for phototropism is a multiple process. The direction of light is detected at the **tip** of the shoot with (b**lue** light is most effective). It is absorbed by a flavoprotein called phototropin. Flavoproteins contain flavin as a prosthetic group. Auxin moves from the tip down. An auxin transporter - one of the **PIN proteins -** is inserted in the plasma membrane at the lateral face of cells of the shoot. Auxin is pumped **out** of these efflux transporters and accumulates in the cells on the shady side. This stimulates elongation of the cells on the shady side causing the shoot to bend toward the light.

Gravitropism

Gravitropism is a plant growth response to gravity.

- Plant **shoots** display **negative** gravitropism: when placed on its side, a plant shoot will grow up
- **Roots** display **positive** gravitropism: they grow down.

Possible Mechanism of Gravitropism in Roots

When a root is placed on its side,

- **Statoliths** (organelles containing starch grains) settle by gravity to the bottom of cells in the **root tip**.
- This causes PIN proteins to redistribute to the underside of the cell where they pump **auxin** out of the cell; that is, they are efflux transporters.
- The auxin then accumulates along the **under** side of the root.
- This INHIBITS root cell elongation.
- So the cells at the **top** surface of the root elongate, causing the root to grow **down**.

Apical Dominance

Growth of the shoot apex (terminal shoot) usually inhibits the development of the lateral buds on the stem beneath. This phenomenon is called *apical dominance*. If the terminal shoot of a plant is removed, the inhibition is lifted, and lateral buds begin growth. Gardeners exploit this principle by pruning the terminal shoot of ornamental shrubs, etc. The release of apical dominance enables lateral branches to develop and the plant becomes bushier. The process usually must be repeated because one or two laterals will eventually outstrip the others and reimpose apical dominance.

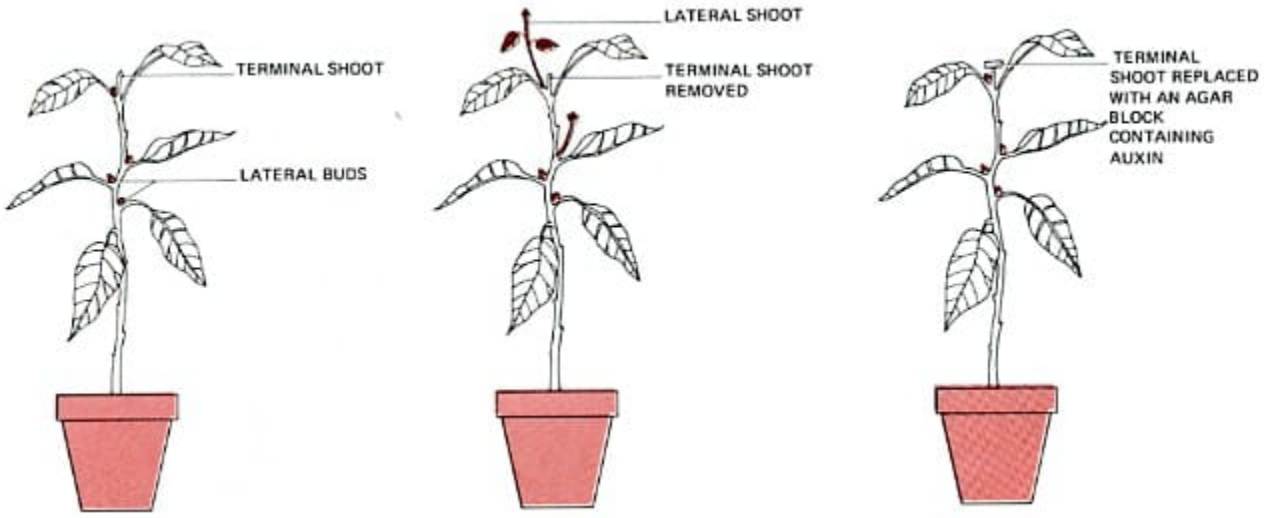


Figure 16.4.3.2.2 Apical dominance

Apical dominance seems to result from the downward transport of auxin produced in the apical meristem. In fact, if the apical

meristem is removed and IAA applied to the stump, inhibition of the lateral buds is maintained.

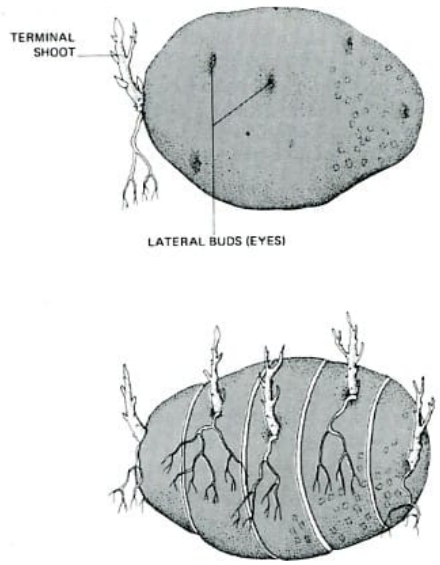


Figure 16.4.3.2.3 Potato eyes

The common white potato is really a portion of the underground stem of the potato plant. It has a terminal bud or "eye" and several lateral buds. After a long period of storage, the terminal bud usually sprouts but the other buds do not. However, if the potato is sliced into sections, one bud to a section, the lateral buds develop just as quickly as the terminal bud.

Fruit Development

Pollination of the flowers of angiosperms initiates the formation of seeds. As the seeds mature, they release auxin to the surrounding flower parts, which develop into the fruit that covers the seeds.

Some commercial growers deliberately initiate fruit development by applying auxin to the flowers. Not only does this ensure that all the flowers will "set" fruit, but it also maximizes the likelihood that all the fruits will be ready for harvest at the same time.

Abscission

Auxin also plays a role in the abscission of leaves and fruits. Young leaves and fruits produce auxin and so long as they do so, they remain attached to the stem. When the level of auxin declines, a special layer of cells — the **abscission layer** — forms at the base of the petiole or fruit stalk. Soon the petiole or fruit stalk breaks free at this point and the leaf or fruit falls to the ground.

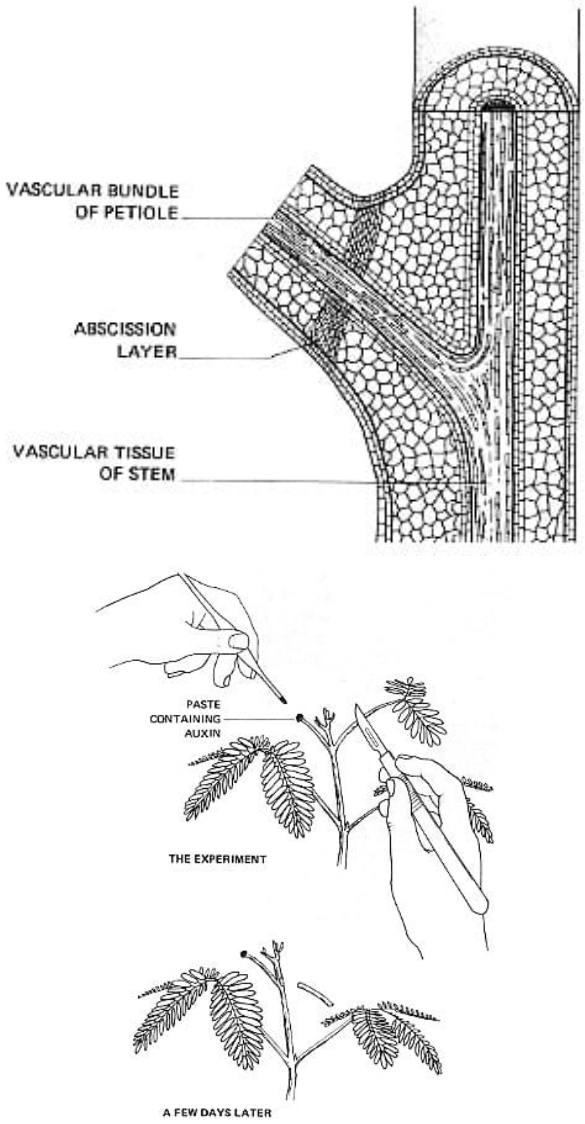


Figure 16.4.3.2.5 Abscission mimosa

The figure on the right shows a nice demonstration of the role of auxin in abscission. If the blade of the leaf is removed, as shown in the figure, the petiole remains attached to the stem for a few more days. The removal of the blade seems to be the trigger as an undamaged leaf at the same node of the stem remains on the plant much longer, in fact, the normal length of time. If, however, auxin is applied to the cut end of the petiole, abscission of the petiole is greatly delayed.

Fruit growers often apply auxin sprays to cut down the loss of fruit from premature dropping.

Root Initiation and Development

The localized accumulation of auxin in epidermal cells of the root initiates the formation of lateral or secondary roots. Auxin also stimulates the formation of **adventitious roots** in many species. Adventitious roots grow from stems or leaves rather than from the regular root system of the plant.

Horticulturists may propagate desirable plants by cutting pieces of stem and placing them base down in moist soil. Eventually adventitious roots grow out at the base of the cutting. The process can often be hastened by treating the cuttings with a solution or powder containing a synthetic auxin.

Once a root is formed, a gradient of auxin concentration develops highest at the tip promoting the production of new cells at the

meristem, and lowest in the region of differentiation, thus promoting the elongation and differentiation of root cells. The drop in auxin activity in the regions of elongation and differentiation is mediated by cytokinin — an auxin antagonist.

Translocation of Auxin

Auxin moves through the plant by two mechanisms: It passes in the sap moving through the phloem from where it is synthesized (its "source", usually the shoot) to a "sink" (e.g., the root). It also passes from cell to cell by the following mechanism.

Auxin can enter the cell by diffusion and also through **influx** transporters in the plasma membrane. It moves out through **efflux** transporters - called **PIN proteins**. Eight different types of PIN proteins have been identified so far. These are transmembrane proteins inserted in localized portions of the plasma membrane, e.g.,

- at the top of the cell where they move auxin toward the top of the plant;
- at the basal surface of the cell where they move auxin down the plant;
- at the lateral surface of the cell where they move auxin laterally (e.g., to mediate phototropism and gravitropism).

Identifying the signals that direct the appropriate placement of the PIN proteins is an active area of research.

How does auxin achieve its many different effects in the plant? Auxin effects are mediated by two different pathways: immediate, direct effects on the cell and turning on of new patterns of gene expression

Direct effects of auxin

The arrival of auxin in the cytosol initiates such immediate responses as

- changes in the concentration of and movement of ions in and out of the cell
- reduction in the redistribution of PIN proteins

Some of the direct effects of auxin may be mediated by its binding to a cell-surface receptor designated **ABP1** ("Auxin-binding protein 1").

Effects of auxin on gene expression

Many auxin effects are mediated by changes in the transcription of genes. Auxin enters the nucleus and binds to its receptor, a protein called **TIR1** ("transport inhibitor response protein 1") which now can bind to proteins responsible for attaching ubiquitin to one or another of several **Aux/IAA proteins**. This triggers the destruction of the Aux/IAA proteins by proteasomes. Aux/IAA proteins normally bind transcription factors called auxin response factors (**ARF**) **preventing** them from activating the promoters and other control sequences of genes that are turned on (or off) by auxin. Destruction of the Aux/IAA proteins **relieves** this inhibition, and gene transcription begins.

This mechanism is another of the many cases in biology where a pathway is turned on by inhibiting the inhibitor of that pathway (a double-negative is a positive). For example, the **gibberellins**, another group of plant hormones, exert their effects using a similar strategy. The presence in the cell of many different Aux/IAA proteins (29 in Arabidopsis), many different ARFs (23 in Arabidopsis) and several (~4) TIR1-like proteins provides a logical basis for mediating the different auxin effects that I have described. But how this is done remains to be discovered.

Synthetic auxins as weed killers

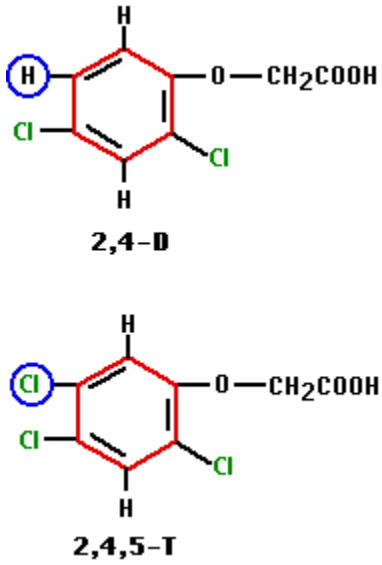


Figure 16.4.3.2.6 2,4-D

Some of the most widely-used weed killers are synthetic auxins. These include **2,4-dichlorophenoxy acetic acid** (**2,4-D**) and **2,4,5-trichlorophenoxy acetic acid** (**2,4,5-T**). As the formulas show, 2,4,5-T is 2,4-D with a third chlorine atom, instead of a hydrogen atom, at the #5 position in the benzene ring (blue circles).

2,4-D and its many variants are popular because they are **selective herbicides**, killing broad-leaved plants but not grasses (no one knows the basis of this selectivity).

Why should a synthetic auxin kill the plant? It turns out that the auxin **influx transporter** works fine for 2,4-D, but that 2,4-D cannot leave the cell through the **efflux transporters**. Perhaps it is the resulting accumulation of 2,4-D within the cell that kills it.

A mixture of 2,4,-D and 2,4,5-T was the "agent orange" used by the U.S. military to defoliate the forest in parts of South Vietnam. Because of health concerns, 2,4,5-T is no longer used in the U.S.

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16.5C: Cytokinins

Cytokinins are plant hormones that are derivatives of the purine adenine. They are not to be confused with cytokines. They were discovered as an absolutely essential ingredient in medium for growing plant cells in culture. Without cytokinins in the medium, plant cells will not divide by mitosis. Cytokinins have been implicated in many plant activities; usually along with some other plant hormone such as auxin or ethylene.

Among these:

- mitosis
- chloroplast development
- differentiation of the shoot meristem
- stimulating the development of lateral buds and therefore branching
- differentiation of the tissues of the root
- leaf formation
- leaf senescence

One of the clearest examples of cytokinin activity occurs in the germination of seeds. The endosperm of monocot seeds, such as corn (maize), contains large stores of the precursor to the cytokinin **zeatin** (right). When the corn kernel germinates, zeatin moves from the endosperm to the root tip where it stimulates vigorous mitosis.



Zeatin

Steps in cytokinin signaling

The following are the steps in cytokinin signaling:

- A cytokinin, like zeatin, binds to a receptor protein embedded in the plasma membrane of the cell.
- The internal portion of the receptor then attaches a phosphate group to a protein in the cytosol.
- This protein moves into the nucleus where
- it activates one or more nuclear transcription factors.
- These bind to the promoters of genes.
- Transcription of these genes produces mRNAs that move out into the cytosol.
- Translation of these mRNAs produces the proteins that enable the cell to carry out its cytokine-induced function.

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16.5D: Ethylene

This page discusses ethylene, a gaseous plant hormone essential for fruit ripening, released as fruits mature. Commercial growers use ethylene generation equipment for uniform ripening. Ethylene is detected by receptors in plant cell ER, triggering a signaling cascade that activates transcription factors and gene expression.

Ethylene is a plant hormone that differs from other plant hormones in being a **gas**.

It has the molecular structure:

$$H_2C{=}CH_2$$

As they approach maturity, many fruits (e.g., apples, oranges, avocados) release ethylene. Ethylene then promotes the **ripening** of the fruit. Commercial fruit growers can buy equipment to generate ethylene so that their harvest ripens quickly and uniformly.

The presence of ethylene is detected by transmembrane receptors in the endoplasmic reticulum (ER) of the cells. Binding of ethylene to these receptors unleashes a signaling cascade that leads to activation of transcription factors and the turning on of gene transcription.

The ill-fated FlavrSavr tomato contains an antisense transgene that interferes with the synthesis of ethylene and hence slows ripening.

Plant functions affected by Ethylene

Ethylene also affects many other plant functions such as:

- abscission of leaves, fruits, and flower petals
- drooping of leaves
- sprouting of potato buds
- seed germination
- stem elongation in rice (by promoting the breakdown of abscisic acid (ABA) and thus relieving ABA's inhibition of gibberellic acid)
- flower formation in some species

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16.5E: Gibberellins

During the 1930s Japanese scientists isolated a growth-promoting substance from cultures of a fungus that parasitizes rice plants. They called it **gibberellin**. After the delay caused by World War II, plant physiologists in other countries succeeded in isolating more than 30 closely-related compounds. One of the most active of these - and one found as a natural hormone in the plants themselves - is **gibberellic acid** (**GA**).

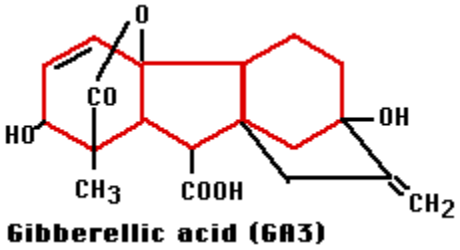


Figure 16.4.3.5.1 GA3

GA has a number of effects on plant growth, but the most dramatic is its effect on **stem growth**. When applied in low concentrations to a bush or "dwarf" bean, the stem begins to grow rapidly. The length of the internodes becomes so great that the plant becomes indistinguishable from climbing or "pole" beans. GA seems to overcome the genetic limitations in many dwarf varieties.

Synthesis of gibberellins also helps grapevines climb up toward the light by causing meristems that would have developed into flowers to develop into **tendrils** instead.

Note

One of the 7 pairs of traits that Mendel studied in peas as he worked out the basic rules of inheritance was dwarf-tall. The recessive gene - today called ***le*** - turns out to encode an enzyme that is defective in enabling the plant to synthesize GA. The dominant gene, ***Le***, encodes a functioning enzyme permitting normal GA synthesis and making the "tall" phenotype.

Effects of gibberellins on gene expression

Gibberellins exert their effects by altering gene transcription, the steps for which are described below.

- Gibberellin enters the cell and binds to a soluble receptor protein called **GID1** ("gibberellin-insensitive dwarf mutant 1") which now can bind to a complex of proteins (**SCF**) responsible for attaching ubiquitin to one or another of several **DELLA proteins**.
- This triggers the destruction of the DELLA proteins by proteasomes.
- DELLA proteins normally bind gibberellin-dependent transcription factors, a prominent one is designated **PIF3/4**, **preventing** them from binding to the DNA of control sequences of genes that are turned on by gibberellin.
- Destruction of the DELLA proteins **relieves** this inhibition and gene transcription begins.

This mechanism is another of the many cases in biology where a pathway is turned on by inhibiting the inhibitor of that pathway (a double-negative is a positive).

Another example: Auxin.

Although most of the specific proteins involved are quite different, both gibberellins and auxin affect gene expression by a similar mechanism of relief of repression.

Ligand	Gibberellins	Auxin
Receptor	GID1	TIR1

Proteasome activator	SCF	SCF
Transcription factor repressor	DELLA	Aux/IAA
Transcription factor	PIF3/4	ARF1

The **dwarf** varieties of **rice** and **wheat** that have played such an important part in the **"green revolution"** carry mutant genes that

- interfere with the synthesis of their gibberellins (in the case of rice)
- for wheat, reduce their ability to respond to their own gibberellins (because of mutant genes for a DELLA protein).

Dwarf varieties of sorghum and more recently maize (corn) also exist, but in these cases, the mutation interferes with **auxin** transport, not gibberellin activity.

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16.5F: Strigolactones

This page discusses strigolactones, root-secreted compounds that aid germination in some seeds, like Striga, which harms crops such as rice and corn by depleting nutrients. They promote beneficial mycorrhizal relationships but aren't classified as plant hormones unless mobility within the plant is confirmed. Recent research indicates that strigolactones also inhibit lateral bud development, thereby regulating branching more specifically than auxin and abscisic acid, which have broader effects.

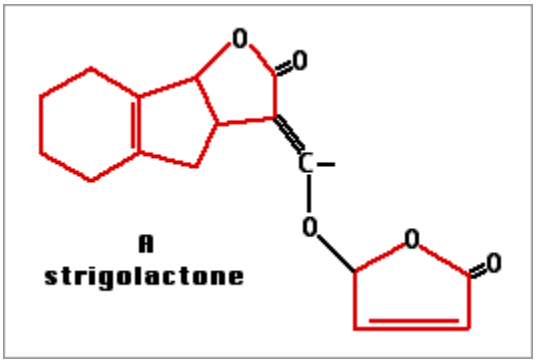


Figure 16.4.3.6.1 Strigolactones

Strigolactones are manufactured in, and secreted from, the roots and have been known for some time to

- promote the germination of some plant seeds. Unfortunately these include seeds of the genus *Striga* (witchweed) whose developing root invades the root of the strigolactone-secreting host plant (e.g., rice, corn, and sorghum) stealing nutrients from it and causing serious crop losses.
- signal mycorrhizal fungi to connect to the root system forming a mutualistic relationship.

However, these activities do not qualify them as plant hormones (both activities take place in the soil surrounding the roots). Only if it can be demonstrated that strigolactones are translocated in the plant from the place of manufacture (roots) to another part of the plant where they exert an effect, can they be called hormones.

Two reports in the 11 September 2008 issue of **Nature** come close to proving the case.

Strigolactones (or possibly molecules derived from them) suppress the development of lateral buds and thus inhibit branching of the plant. Mutations in genes needed for the synthesis of strigolactones stimulate the development of lateral buds producing a more highly-branched plant than normal. Application of a synthetic strigolactone near the base of these mutant plants inhibits development of lateral buds above and thus restores normal branching.

Auxin and, in certain circumstances, abscisic acid also inhibit branching, that is, they promote apical dominance. But both auxin and abscisic acid participate in a number of different plant functions while the effect of strigolactones on branching seems quite specific.

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UNIT 17: ECOLOGY

Ecology studies the interactions among organisms and their environment. Objects of study include interactions of organisms with each other and with abiotic components of their environment. Topics of interest include the biodiversity, distribution, biomass, and populations of organisms, as well as cooperation and competition within and between species. Ecosystems are dynamically interacting systems of organisms, the communities they make up, and the non-living components of their environment. Ecosystem processes, such as primary production, pedogenesis, nutrient cycling, and niche construction, regulate the flux of energy and matter through an environment.

17.1: Energy Flow through the Biosphere

17.1A: Ecosystem Productivity

17.1B: Food Chains and Food Webs

17.1C: Biomes

17.1D: Freshwater Ecosystems

17.1E: Marine Ecosystems

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17.2: Cycles of Matter in the Biosphere

17.2A: Carbon Cycle

17.2B: Nitrogen Cycle

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17.2D: Soil

17.2E: Sewage Treatment

17.2F: Chlorination and the Law of Unintended Consequences

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17.2H: Acid Rain

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17.3: The Growth of Populations

17.3A: The Human Population

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17.4: Interactions between Species

17.4A: Symbiosis

17.4B: Insecticides

17.4C: Biological Control of Pests

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17.1: ENERGY FLOW THROUGH THE BIOSPHERE



- Topic hierarchy

17.1A: Ecosystem Productivity

17.1B: Food Chains and Food Webs

17.1C: Biomes

17.1D: Freshwater Ecosystems

17.1E: Marine Ecosystems

17.1F: Biomagnification of Pesticides

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17.1A: Ecosystem Productivity

This page discusses the energy availability in tropical regions, where daily energy received is 8,000 to 10,000 kilocalories per square meter, with only 2.2% trapped by plants. Net productivity indicates energy stored post-respiration, showing variances among ecosystems such as tropical rainforests at 15,000 kcal/m²/year. Humans utilize about 20% of this net productivity for resources, and activities like urban development and deforestation diminish global productivity.

The Input of Energy

Tropical regions every day and temperate regions during the growing season receive some 8,000 to 10,000 kilocalories (kcal) of energy each day on each square meter (1 m^2) of surface. A kilocalorie is the amount of heat needed to warm 1 kg of water 1 degree Celsius (°C). Because all of the light trapped in photosynthesis is ultimately released as heat, it makes sense to follow the flow of energy through ecosystems in units of heat.

How efficient are plants at converting this energy into organic molecules?

Gross Productivity

Gross productivity is the amount of energy trapped in organic matter during a specified interval at a given trophic level.

The table shows the use of visible sunlight is a cattail marsh. The plants have trapped only 2.2% of the energy falling on them.

Photosynthesis	2.2%
Reflection	3.0
Evaporation (including transpiration and heating of the surroundings	94.8
Total	100.0%

However, at least half of this is lost by cellular respiration as the plants run their own metabolism.

Net Productivity

Net productivity is the amount of energy trapped in organic matter during a specified interval at a given trophic level **less** that lost by the respiration of the organisms at that level. One way to determine this is to collect and weigh the plant material produced on 1 m^2 of land over a given interval. One gram of plant material (e.g., stems and leaves), which is largely carbohydrate, yields about 4.25 kcal of energy when burned (or respired).

The table shows representative values for the net productivity of a variety of ecosystems — both natural and managed. These values are only approximations and are subject to marked fluctuations because of variations in temperature, fertility, and availability of water.

Estimated Net Productivity of Certain Ecosystems (in kilocalories/m^2/year)	
Temperate deciduous forest	5,000
Tropical rain forest	15,000
Tall-grass prairie	2,000
Desert	500
Coastal marsh	12,000

Ocean close to shore	2,500
Open ocean	800
Clear (oligotrophic) lake	800
Lake in advanced state of eutrophication	2,400
Silver Springs, Florida	8,800
Field of alfalfa (lucerne)	15,000
Corn (maize) field, U.S.	4,500
Rice paddies, Japan	5,500
Lawn, Washington, D.C.	6,800
Sugar cane, Hawaii	25,000

What happens to the net productivity of a plant community?

- Some is harvested by plant-eating **herbivores**, e.g., insects, deer. Often this is only the start of a series of transformations as it passes through a series of heterotrophs — that together make up a **food chain**.
- Some is consumed by organisms of decay, e.g., fungi and bacteria.
- Some may be **stored**, e.g., in a growing forest or as peat in a bog.

What about humans?

Humans, like all heterotrophs, depend upon net productivity for their food both directly as we consume plant material (e.g., rice, wheat, corn) and indirectly when we eat animals that have, themselves, fed on plant material (poultry, cattle, sheep, etc.) and/or animal products (e.g., milk, eggs).

We also use the earth's net productivty to meet other needs such as:

- wood for fuel
- wood and fiber (e.g., cotton, flax) to house and clothe us

Added together it is estimated that our species now appropriates some 20% of world's net productivity for our own use. However, this figure obscures large regional variations with estimates running as high as 80% in South central Asia to as low as 6% in South America.

We also reduce the net productivity of our planet by other activities such as

- paving over land for buildings, roads, parking lots, etc.
- burning forests to clear them for agriculture

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17.1B: Food Chains and Food Webs

The source of all food is the activity of autotrophs, mainly photosynthesis by plants. They are called **producers** because only they can manufacture food from inorganic raw materials. This food feeds **herbivores**, called **primary consumers**. **Carnivores** that feed on herbivores are called **secondary consumers**. Carnivores that feed on other carnivores are **tertiary** (or higher) consumers. Such a path of food consumption is called a **food chain**. Each level of consumption in a food chain is called a **trophic level**.

The table gives one example of a food chain and the trophic levels represented in it.

Grass →	**Grasshopper** →	**Toad** →	**Snake** →	**Hawk** →	**Bacteria of decay**
In general,					
Autotrophs (Producers) →	Herbivores (Primary Consumers) →	Carnivores (Secondary, tertiary, etc. consumers) →			Decomposers

Food Webs

Most food chains are interconnected. Animals typically consume a varied diet and, in turn, serve as food for a variety of other creatures that prey on them. These interconnections create **food webs**.

Energy Flow Through Food Chains

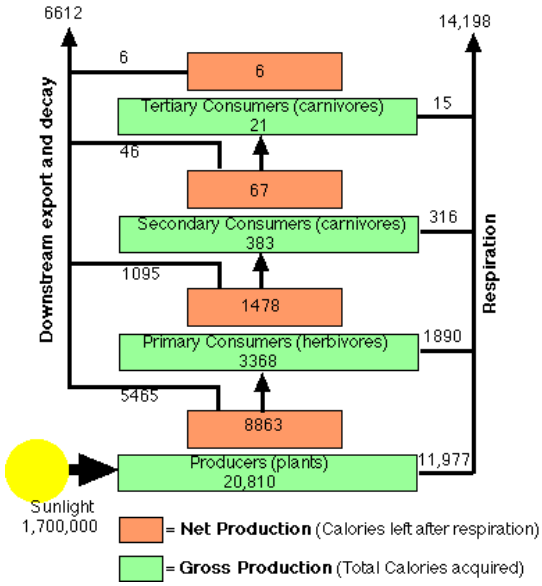


Figure 17.1.2.1 Silver Springs ecosystem

H. T. Odum analyzed the flow of energy through a river ecosystem in Silver Springs, Florida. His findings are shown here. The figures are given in **kilocalories per square meter per year** (**kcal/m^2/yr**).

At each trophic level,

- Net production is only a fraction of gross production because the organisms must expend energy to stay alive. Note that the difference between gross and net production is greater for animals than for the producers - reflecting their greater activity.
- Much of the energy stored in net production was lost to the system by

    - decay
    - being carried downstream
- Note the substantial losses in net production as energy passes from one trophic level to the next.
- The ratio of net production at one level to net production at the next higher level is called the **conversion efficiency**. Here it varied from
    - 17% from producers to primary consumers (1478/8833) to
    - 4.5% from primary to secondary consumers (67/1478).
- From similar studies in other ecosystems, we can take 10% as the average conversion efficiency from producers to primary consumers.

  Animal husbandry often exceeds this 10% value. For example, broilers (young chickens) can gain half a pound (227 g) of weight for every pound (454 g) of food they eat. (Since the water content of the two is not the same, the conversion efficiency is somewhat less than the apparent 50%.) Nonetheless, the loss of energy as it passes from producers to primary consumers explains, for example, why it costs more to buy a pound of beefsteak than a pound of corn.

  Conversion efficiencies from primary consumers to secondary consumers (herbivores to carnivores) tend to be much lower, averaging about 1%.

- In this ecosystem, all the gross production of the producers (20,810) ultimately disappeared in respiration (14,198) and downstream export and decay (6612). So there was no storage of energy from one year to the next. This is typical of mature ecosystems, such as a mature forest.

Some ecosystems do store energy, for example,

- The slow rate of decay in bogs causes peat to accumulate (the source of the world's coal)
- A young forest accumulates organic matter as the trees grow.

The Pyramid of Energy

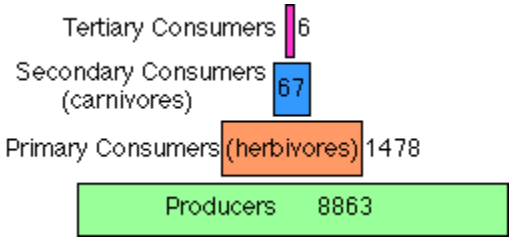


Figure 17.1.2.2 Silver Springs

Conversions efficiencies are always much less than 100%. At each link in a food chain, a substantial portion of the sun's energy — originally trapped by a photosynthesizing autotroph - is dissipated back to the environment (ultimately as heat). Thus it follows that the total amount of energy stored in the bodies of a given population is dependent on its trophic level. For example, the total amount of energy in a population of toads must necessarily be far less than that in the insects on which they feed. The insects, in turn, have only a fraction of the energy stored in the plants on which they feed. This decrease in the total available energy at each higher trophic level is called the **pyramid of energy**.

Using Odum's data on net productivity at the various levels in Silver Springs, we get this pyramid. The figures represent net production at each trophic level expressed in **kcal/m^2/yr**.

The Pyramid of Biomass

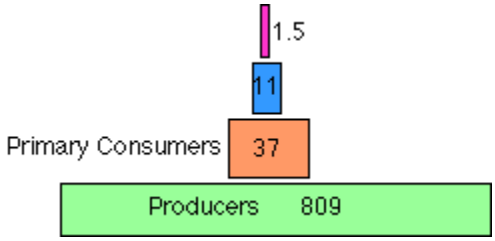


Figure 17.1.2.3 Silver Springs

How does one measure the amount of energy in a population?

Since all organisms are made of roughly the same organic molecules in similar proportions, a measure of their dry weight is a rough measure of the energy they contain. A census of the population, multiplied by the weight of an average individual in it, gives an estimate of the weight of the population. This is called the **biomass** (or **standing crop**). This, too, diminishes with the distance along the food chain from the autotrophs which make the organic molecules in the first place.

The graphic shows the pyramid of biomass for Silver Springs. (It, too, is based on the data obtained by Howard T. Odum.) The figures represent the dry weight of organic matter (per square meter) at the time of sampling. Analysis of various ecosystems indicates that those with squat biomass pyramids (with conversion efficiencies between one trophic level and the next averaging 10% or better) are less likely to be disrupted by physical or biotic changes than those with tall, skinny pyramids (having conversion efficiencies less than 10%).

The Pyramid of Numbers

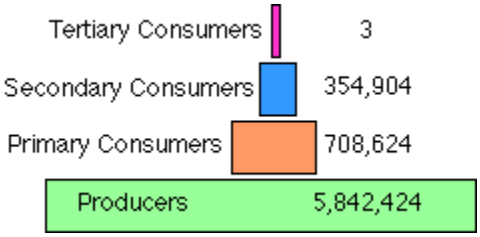


Figure 17.1.2.4 Bluegrass pyramid

Small animals are more numerous than larger ones. This graph shows the pyramid of numbers resulting when a census of the populations of autotrophs, herbivores, and two levels of carnivores was taken on an acre (0.4 hectare) of grassland. The figures represent **number of individuals** counted at each trophic level. The pyramid is based on data acquired by Evans, Cain, and Walcott, and has been redrawn by permission from E. P. Odum, **Fundamentals of Ecology**, 2nd. ed., © W. B. Saunders Co., Philadelphia, 1959.

The pyramid arises because:

- Each species is limited in its total biomass by its trophic level.
- So, if the size of the individuals at a given trophic level is small, their numbers can be large and vice versa.
- Predators are usually larger than their prey.
- Occupying a higher trophic level, their biomass must be smaller.
- Hence, the number of individuals in the predator population is much smaller than that in the prey population.

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17.1C: Biomes

A biome is a large, distinctive complex of plant communities created and maintained by **climate**.

How many biomes are there?

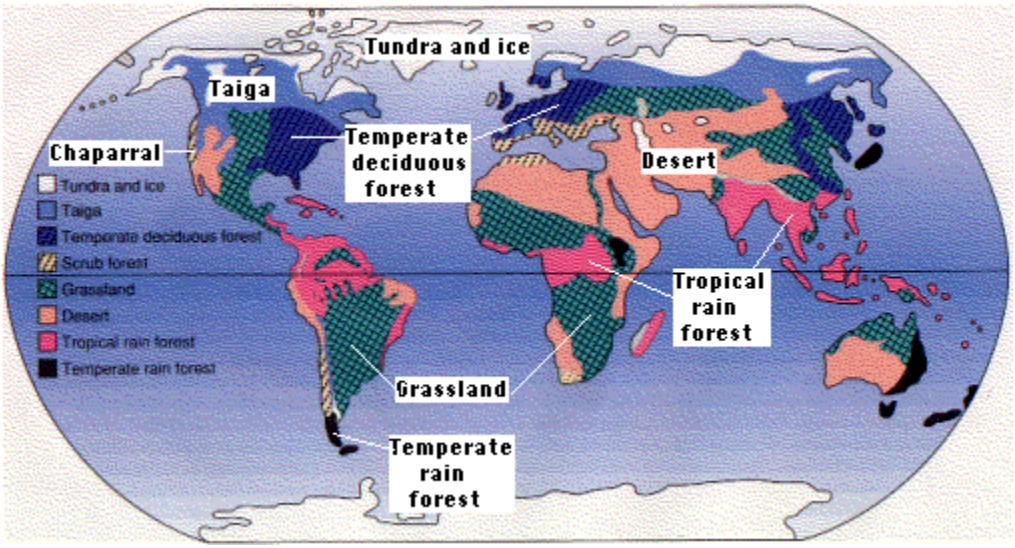


Figure 17.1.3.1 Biomes

A study published in 1999 concluded that there are 150 different "ecoregions" in North America alone. But I shall cast my lot with the "lumpers" rather than the "splitters" and lump these into **8 biomes**:

- tundra
- taiga
- temperate deciduous forest
- scrub forest (called chaparral in California)
- grassland
- desert
- tropical rain forest
- temperate rain forest

The figure shows the distribution of these 8 biomes around the world. A number of climatic factors interact in the creation and maintenance of a biome. Where precipitation is moderately abundant — 40 inches (about 1 m) or more per year — and distributed fairly evenly throughout the year, the major determinant is **temperature**. It is not simply a matter of average temperature, but includes such limiting factors as whether it ever freezes or length of the growing season. If there is ample rainfall, we find 4 characteristic biomes as we proceed from the tropics (high temperatures) to the extreme latitudes (low temperatures). In order, they are:

- tropical rain forest or jungle
- temperate deciduous forest
- taiga
- tundra

Tropical Rain Forest



Figure 17.1.3.2 Rainforest

In the Western Hemisphere, the tropical rain forest reaches its fullest development in the jungles of Central and South America.

- The trees are very tall and of a great variety of species.
- One rarely finds two trees of the same species growing close to one another.
- The vegetation is so dense that little light reaches the forest floor.
- Most of the plants are evergreen, not deciduous.
- The branches of the trees are festooned with vines and epiphytes (see the photo taken in the Luquillo National Forest of Puerto Rico).

Note

Epiphytes are plants that live perched on sturdier plants. They do not take nourishment from their host as parasitic plants do. Because their roots do not reach the ground, they depend on the air to bring them moisture and inorganic nutrients. Many orchids and many bromeliads (members of the pineapple family like "Spanish moss") are epiphytes.

The lushness of the tropical rain forest suggests a high net productivity, but this is illusory. Many of the frequent attempts to use the tropical rain forest for conventional crops have been disappointing. Two problems:

- The high rainfall leaches soil minerals below the reach of plant roots.
- The warmth and moisture cause rapid decay so little humus is added to the soil.

The tropical rain forest exceeds all the other biomes in the diversity of its animals as well as plants. Most of the animals - mammals and reptiles, as well as birds and insects live in the trees. The closest thing to a tropical rain forest in the continental United States are the little wooded "islands" found scattered through the Everglades in the southern tip of Florida. Their existence depends on the fact that it never freezes, and they often escape the fires that periodically sweep the Everglades.

Temperate Deciduous Forest



Figure 17.1.3.3 Temperate forest

This biome occupies the eastern half of the United States and a large portion of Europe. It is characterized by:

- hardwood trees (e.g., beech, maple, oak, hickory) which
- are deciduous; that is, shed their leaves in the autumn.
- The number of different species is far more limited than in the jungle.
- Large stands dominated by a single species are common.
- Deer, raccoons, and salamanders are characteristic inhabitants.
- During the growing season, this biome can be quite productive in both natural and agricultural ecosystems.

The photo (by Dick Morton) shows a view of this biome in Maine in the autumn.

Taiga



Fig.17.1.3.4 Taiga

The taiga is named after the biome in Russia.

- It is a land dominated by **conifers**, especially spruces and firs.
- It is dotted with lakes, bogs, and marshes.
- It is populated by an even more limited variety of plants and animals than is the temperate deciduous forest.
- In North America, the moose is such a typical member that it has led to the name: "spruce-moose" biome.
- Before the long, snowy winter sets in, many of the mammals hibernate, and many of the birds migrate south.
- Although the long days of summer permit plants to grow luxuriantly, net productivity is low.

The photo (courtesy of Dr. Benjamin Dane of Tufts University) shows the "spruce-moose" biome in British Columbia.

Tundra

At extreme latitudes, the trees of the taiga become stunted by the harshness of the subarctic climate. Finally, they disappear leaving a land of bogs and lakes.

- The climate is so cold in winter that even the long days of summer are unable to thaw the **permafrost** beneath the surface layers of soil.
- Sphagnum moss, a wide variety of lichens, and some grasses and fast-growing annuals dominate the landscape during the short growing season.
- Caribou feed on this growth as do vast numbers of insects.
- Swarms of migrating birds, especially waterfowl, invade the tundra in the summer to raise their young, feeding them on a large variety of aquatic invertebrates and vertebrates.
- As the brief arctic summer draws to a close, the birds fly south, and
- all but a few of the permanent residents, in one way or another, prepare themselves to spend the winter in a dormant state.

Biomes established by altitude

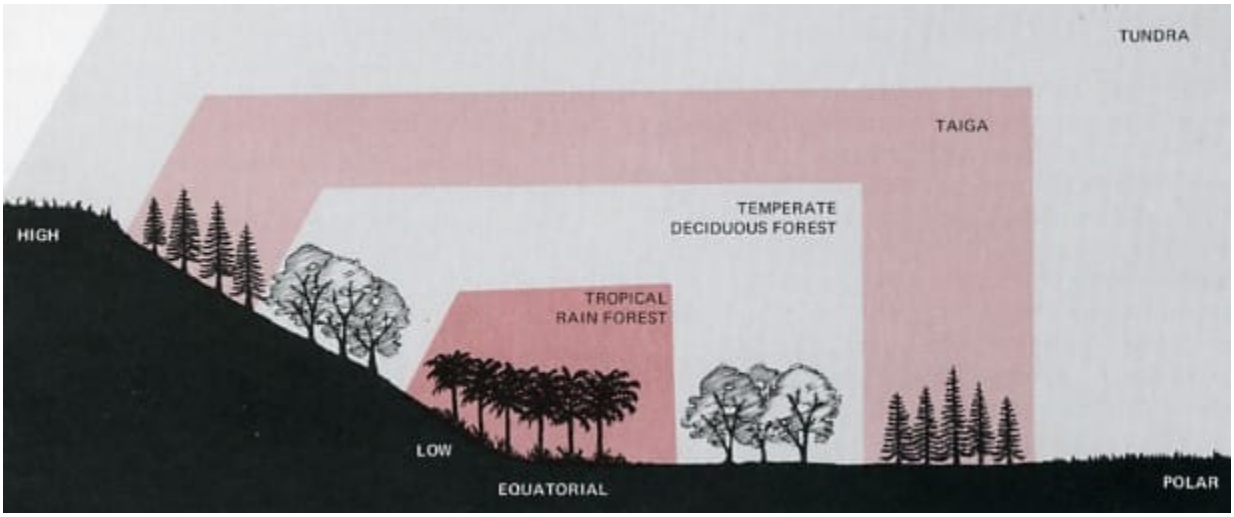


Figure 17.1.3.5 Biomes Altitude vs Latitude

Temperature is the major influence on the biomes discussed above. Because temperatures decline with altitude as well as latitude, similar biomes exist on mountains even when they are at low latitudes. As a rule of thumb, a climb of 1000 feet (about 300 m) is equivalent in changed flora and fauna to a trip northward of some 600 miles (966 km).



Figure 17.1.3.6 Alpine tundra

The photo is of alpine tundra at 12,000 feet (3,658 m) in the Rocky Mountains.

Field studies in various parts of the Northern Hemisphere have shown that in recent decades many species of animals and plants have

- shifted their ranges farther north (averagiing 16.9 kilometers per decade)
- shifted their ranges higher in the mountains (averaging 11.0 meters per decade)

These observations add to the growing body of evidence that global warming is affecting a broad assortment of living things.

Biomes established by rainfall

The other major biomes are controlled not so much by temperature but by the amount and seasonal distribution of rainfall.

The prevailing winds in the western half of North America blow in from the Pacific laden with moisture. Each time this air rises up from the western slopes of, successively, the Coast Ranges, the Sierras and Cascades, and finally the Rockies, it expands and cools. Its moisture condenses to rain or snow, which drenches the mountain slopes beneath. When the air reaches the eastern slopes, it is relatively dry, and much less precipitation falls. How much falls and when determine whether the biome will be

- temperate rain forest
- grassland
- desert or
- chaparral

Temperate Rain Forest

The temperate rain forest combines high annual rainfall with a temperate climate. The Olympic Peninsular in North America is a good example. An annual rainfall of as much as 150 inches (381 cm) produces a lush forest of conifers.

Grasslands



Figure 17.1.3.7 Grasslands

Grasslands are also known as **prairie** or **plains**. The annual precipitation in the grasslands averages 20 inches (~51 cm) per year. A large proportion of this falls as rain early in the growing season. This promotes a vigorous growth of perennial grasses and herbs, except along river valleys - is barely adequate for the growth of forests. The photo shows grassland in the Badlands National Monument in South Dakota.

Fire is probably the factor that tips the balance from forest to grasslands. Fires set by lightning and by humans regularly swept the plains in earlier times. Thanks to their underground stems and buds, perennial grasses and herbs are not harmed by fires that destroy most shrubs and trees.

The abundance of grass for food, coupled with the lack of shelter from predators, produces similar animal populations in grasslands throughout the world. The dominant vertebrates are swiftly-moving, herbivorous ungulates. In North America, bison and antelope were conspicuous members of the grassland fauna before the coming of white settlers. Now the level grasslands supply corn, wheat, and other grains, and the hillier areas support domesticated ungulates: cattle and sheep.

When cultivated carefully, the grassland biome is capable of high net productivity. A major reason: rainfall in this biome never leaches soil minerals below the reach of the roots of crop plants.

Desert



Figure 17.1.3.8 Desert

Annual rainfall in the desert is less than 10 inches (25 cm) and, in some years, may be zero. Because of the extreme dryness of the desert, its colonization is limited to

- plants such as cacti, sagebrush, and mesquite that have a number of adaptations that conserve water over long periods
- fast-growing annuals whose seeds can germinate, develop to maturity, flower, and produce a new crop of seeds all within a few weeks following a rare, soaking rain.

The photo shows the desert in the Anza-Borego park in southern California.

Many of the animals in the desert (mammals, lizards and snakes, insects, and even some birds) are adapted for burrowing to escape the scorching heat of the desert sun. Many of them limit their forays for food to the night. The net productivity of the desert is low. High productivity can sometimes be achieved with **irrigation**, but these gains are often only temporary. The high rates of evaporation cause minerals to accumulate near the surface and soon their concentration may reach levels toxic to plants.

Chaparral



Figure 17.1.3.8 Chaparral

The annual rainfall in the chaparral biome may reach 20–30 inches (64–76 cm), but in contrast to the grasslands, almost all of this falls in winter. Summers are very dry and all the plants - trees, shrubs, and grasses - are more or less dormant then.

The chaparral is found in California. (The photo shows the chaparral-clad foothills of the Sierra Nevada in California.) Similar biomes (with other names, such as scrub forest), are found around much of the Mediterranean Sea and along the southern coast of Australia.

The trees in the chaparral are mostly oaks, both deciduous and evergreen. Scrub oaks and shrubs like manzanita and the California lilac (not a relative of the eastern lilac) form dense, evergreen thickets. All of these plants are adapted to drought by such mechanisms as waxy, waterproof coatings on their leaves. The chaparral has many plants brought to it from similar biomes elsewhere. Vineyards, olives, and figs flourish just as they do in their native Mediterranean biome. So, too, do eucalyptus trees transplanted from the equivalent biome in Australia.

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17.1D: Freshwater Ecosystems

This page explains that only 3% of the world's water is fresh, largely found in frozen or buried forms. Freshwater ecosystems include the littoral, limnetic, and profundal zones in lakes, each supporting different life forms and productivity levels. Seasonal changes lead to oxygen mixing in these zones. Rivers and streams typically have higher oxygen levels and rely less on photosynthesis, while oceans have similar zones but different naming conventions.

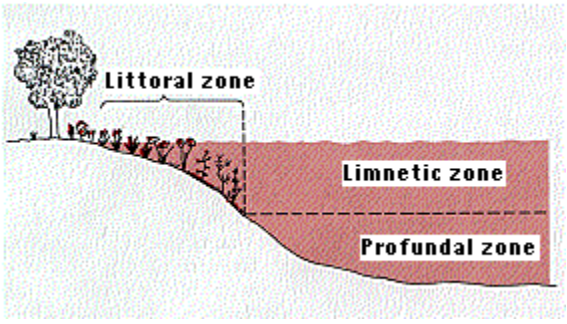


Figure 17.1.4.1 Littoral zone

The zone close to shore. Here light reaches all the way to the bottom. The producers are plants rooted to the bottom and algae attached to the plants and to any other solid substrate. The consumers include

- tiny crustaceans
- flatworms
- insect larvae
- snails
- frogs, fish, and turtles.

Limnetic zone

This is the layer of open water where photosynthesis can occur. As one descends deeper in the limnetic zone, the amount of light decreases until a depth is reached where the rate of photosynthesis becomes equal to the rate of respiration. At this level, net primary production no longer occurs.

The limnetic zone is shallower in turbid water than in clear and is a more prominent feature of lakes than of ponds.

Life in the limnetic zone is dominated by

- floating microorganisms - called **plankton**
- actively swimming animals - called **nekton**

The **producers** in this ecosystem are planktonic algae. The **primary consumers** include such animals as microscopic crustaceans and rotifers - the so-called **zooplankton**. The secondary (and higher) consumers are swimming insects and fish. These nekton usually move freely between the littoral and limnetic zones.

Profundal zone

Many lakes (but few ponds) are so deep that not enough light reaches here to support net primary productivity. Therefore, this zone depends for its calories on the drifting down of organic matter from the littoral and limnetic zones. The profundal zone is chiefly inhabited by primary consumers that are either attached to or crawl along the sediments at the bottom of the lake. Such bottom-dwelling animals are called the **benthos**. The sediments underlying the profundal zone also support a large population of bacteria and fungi. These decomposers break down the organic matter reaching them, releasing inorganic nutrients for recycling.

Fall overturn

Where there is a pronounced change of seasons, the warming of the surface of the lake in the summer prevents this water from mixing with deeper water. This is because warm water is less dense than cold.

The surface water becomes enriched in oxygen some from the air above it and the rest - because it is in the limnetic zone - from photosynthesis. But the water in the profundal zone - eing removed from both these sources - becomes stagnant. In the fall, however, as the surface water cools, it becomes denser and sinks to the bottom — carrying oxygen with it.

Spring overturn

A similar phenomenon occurs when the ice melts in the spring.

Rivers and Streams

The habitats available in rivers and streams differ in several ways from those in lakes and ponds.

- Because of the current, the water is usually more oxygenated.
- Photosynthesizers play a minor role in the food chains here; a large fraction of the energy available for consumers is brought from the land; e.g., in falling leaves.

Oceans, like lakes, can be described in terms of zones. There are many parallels between the two but unfortunately a separate vocabulary is used for each.

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17.1E: Marine Ecosystems

Intertidal zone

Examples:

- sandy beaches
- rocks
- estuaries
- mangrove swamps
- coral reefs
- coastal marshes

Some of these regions are quite productive. Many of their inhabitants have adaptations that enable them to survive periodic exposure to the air and wave action.

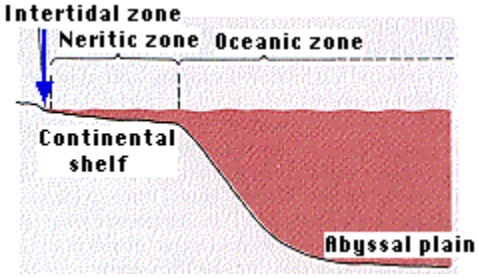


Figure 17.1.5.1 Ocean

Neritic zone

This is the relatively shallow ocean that extends to the edge of the continental shelf. Net productivity here depends on planktonic algae growing as deep as the light can reach.

Oceanic zone

Located over the ocean basins. Here, too, net productivity is pretty much limited to the depths that light can reach. The producers are planktonic algae that support secondary and higher consumers (e.g., fish) in the **nekton**.

Despite its diversity of life, the net productivity of the open ocean is little better than that of a desert.

Abyssal plain

The bottom of the ocean basins: This dark, relatively unvarying region is largely inhabited by sparse populations of bottom-dwelling organisms that make up the **benthos**. These are consumers and decomposers who depend on the organic matter drifting down from the upper portions of the sea.

An exception: the communities around rifts. Rifts are spreading cracks in the sea floor where **continental drift** is taking place. Although no light reaches here, net productivity does occur.

Chemoautotrophic bacteria and archaea manufacture food using energy secured by oxidizing the sulfur flowing out of the cracks ("black smokers"). These microbes support a large population of animals, e.g., tube worms. Some of these worms harbor chemoautotrophic microbes within their tissues which probably supply them with the bulk of their calories.

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17.1F: Biomagnification of Pesticides

The figure shows how DDT becomes concentrated in the tissues of organisms representing four successive trophic levels in a food chain. The concentration effect occurs because DDT is metabolized and excreted much more slowly than the nutrients that are passed from one trophic level to the next. So DDT accumulates in the bodies (especially in fat). Thus most of the DDT ingested as part of gross production is still present in the net production that remains at that trophic level.

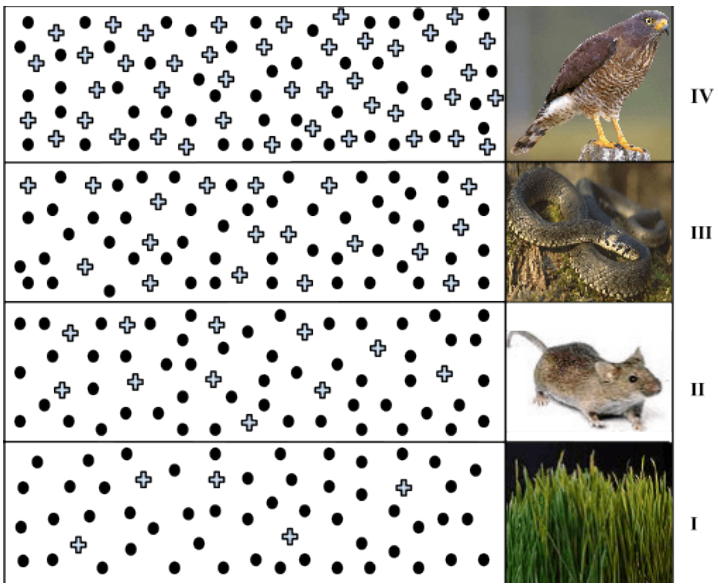


Figure 17.1.6.1: In biomagnification the concentration of the persistent toxins (crosses) increase higher up the food chain. An increase of toxin concentration as the food chain moves up to higher levels. Organisms at the top have a higher tissue concentration of toxins and pollutants than lower levels. The concentration system is due to persistence of the toxins, food chain energetics, and low rate of internal degradation or excretion of the substance.Trophic level I represents the primary producers. Trophic level II represents the primary consumers. Trophic level III represents the secondary consumers. Trophic level IV represents the tertiary consumers. (CC CC-SA 3.0).

This is why the hazard of DDT to nontarget animals is particularly acute for those species living at the top of food chains.

For example,

- spraying a marsh to control mosquitoes will cause trace amounts of DDT to accumulate in the cells of microscopic aquatic organisms, the plankton, in the marsh.
- In feeding on the plankton, filter-feeders, like clams and some fish, harvest DDT as well as food. (Concentrations of DDT 10 times greater than those in the plankton have been measured in clams.)
- The process of concentration goes right on up the food chain from one trophic level to the next. Gulls, which feed on clams, may accumulate DDT to 40 or more times the concentration in their prey. This represents a 400-fold increase in concentration along the length of this short food chain.

There is abundant evidence that some carnivores at the ends of longer food chains (e.g. ospreys, pelicans, falcons, and eagles) suffered serious declines in fecundity and hence in population size because of this phenomenon in the years before use of DDT was banned (1972) in the United States.

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17.2: CYCLES OF MATTER IN THE BIOSPHERE

- Topic hierarchy

17.2A: Carbon Cycle

17.2B: Nitrogen Cycle

17.2C: Symbiotic Nitrogen Fixation

17.2D: Soil

17.2E: Sewage Treatment

17.2F: Chlorination and the Law of Unintended Consequences

17.2G: Air Pollution

17.2H: Acid Rain

17.2I: Ozone

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17.2A: Carbon Cycle

The concentration of carbon in living matter (18%) is almost 100 times greater than its concentration in the earth (0.19%). So living things extract carbon from their nonliving environment. For life to continue, this carbon must be recycled. Carbon exists in the nonliving environment as:

- carbon dioxide (CO_2) in the atmosphere and dissolved in water (forming HCO_3^-)
- carbonate rocks (limestone and coral = $CaCO_3$)
- deposits of coal, petroleum, and natural gas derived from once-living things
- dead organic matter, e.g., humus in the soil

Carbon enters the biotic world through the action of primarily **photoautotrophs**, like plants and algae, that use the energy of light to convert carbon dioxide to organic matter and to a small extent, **chemoautotrophs -** bacteria and archaea that do the same but use the energy derived from an oxidation of molecules in their substrate. Carbon returns to the atmosphere and water by respiration (as CO_2), burning, and decay (producing CO_2 if oxygen is present, methane (CH_4) if it is not.

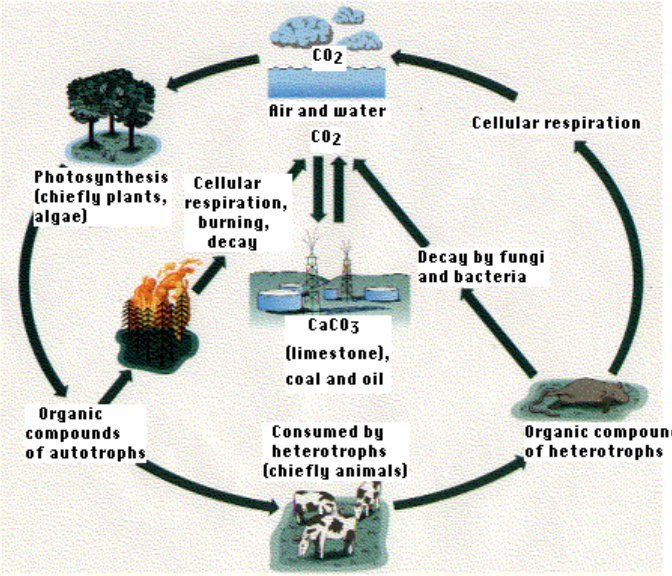


Figure 17.2.1.1 Carbon cycle

The uptake and return of CO_2 are not in balance

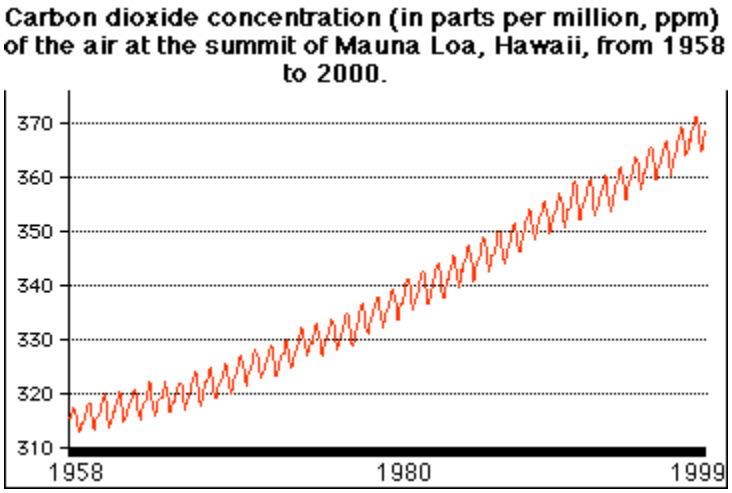


Figure 17.2.1.2 Carbon dioxide concentration

The carbon dioxide content of the atmosphere is gradually and steadily increasing. The graph shows the CO_2 concentration at the summit of Mauna Loa in Hawaii from 1958 through 1999. The values are in parts per million (ppm). The seasonal fluctuation is caused by the increased uptake of CO_2 by plants in the summer. (In March 2015, its average worldwide concentration reached 400 ppm.)

The increase in CO_2 probably began with the start of the industrial revolution. Samples of air trapped over the centuries in the glacial ice of Greenland show no change in CO_2 content until 300 years ago. Since measurements of atmospheric CO_2 began late in the nineteenth century, its concentration has risen over 20%. This increase is surely "anthropogenic"; that is, caused by human activities:

- burning fossil fuels (coal, oil, natural gas) which returns to the atmosphere carbon that has been locked within the earth for millions of years.
- clearing and burning of forests, especially in the tropics. In recent decades, large areas of the Amazon rain forest have been cleared for agriculture and cattle grazing.

Where is the missing carbon?

Curiously, the increase in atmospheric CO_2 is only about one-half of what would have been expected from the amount of fossil fuel consumption and forest burning. Where has the rest gone? Research has shown that increased CO_2 levels lead to increased net production by photoautotrophs. There is evidence that at least some of the missing CO_2 has been incorporated by: (1) increased growth of forests, especially in North America, (2) increased amounts of photoautotrophic plankton in the oceans, and (3) uptake by desert soils (mechanism as yet unknown)

The Greenhouse Effect and Global Warming

Despite these "sinks" for our greatly-increased CO_2 production, the concentration of atmospheric CO_2 continues to rise? Should we be worried? Carbon dioxide is transparent to light but rather opaque to heat rays. Therefore, CO_2 in the atmosphere retards the radiation of heat from the earth back into space - the "greenhouse effect". Has the increase in carbon dioxide led to global warming?

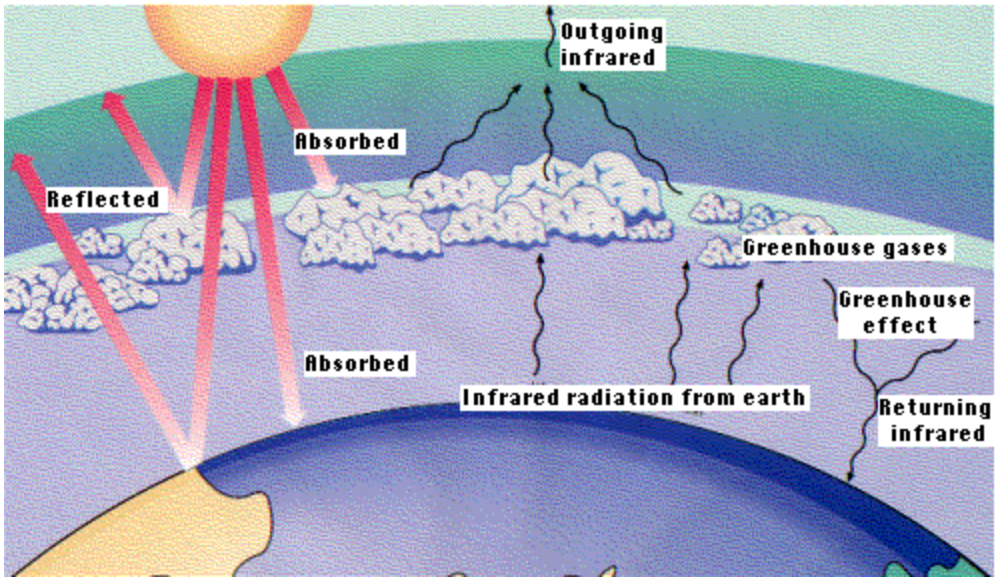


Figure 17.2.1.3 Green house

Some evidence:

- Careful monitoring shows that the global air temperature in 2014 was 0.57°C higher than the average from 1961–1990, and that 14 of the 15 warmest years since records began being kept late in the 19th Century have occurred in this century (including 2005 and 2010 as well as 2014).
- Many glaciers and ice sheets are receding.
- Woody shrubs are now growing in areas of northern Alaska that 50 years ago were barren tundra.
- Many angiosperms in temperate climates are flowering earlier in the spring than they used to.
- Many species of birds and butterflies are moving north and breeding earlier in the spring.

Will continued increase in carbon dioxide lead to more global warming and, if so, how much? At this point, the answer depends on what assumptions you plug into your computer models. But as the different models have been improved, they seem to be converging on a consensus: a doubling of the CO_2 concentration (expected by the end of this century) will cause the earth to warm somewhere in the range of 1.1–6.4°C.

Other Greenhouse Gases

Although their levels in the atmosphere are much lower than that of CO_2,

- **methane** (CH_4)
- **nitrous oxide** (N_2O)
- hydrofluorocarbons (HFCs)

are also potent greenhouse gases.

Methane

Although methane ("marsh gas") is released by natural processes (e.g. from decay occurring in swamps), human activities now account for some 60% of the total.

- mining, processing, and use of coal, oil, and natural gas
- release from landfills
- growing rice in paddies
- burning forests
- raising cattle (fermentation in their rumens produces methane that is expelled from their GI tract)

So burning of the tropical rain forest adds to the atmospheric methane budget in two ways:

- incomplete combustion during burning
- release from the GI tract of the cattle that are later placed on the cleared land.

The methane concentration in Arctic air is presently some 1.9 parts per million, the highest level seen such measurements began. Although this concentration is far less than that of CO_2, methane is 28 times as potent a greenhouse gas. The marked warming of the earth that occurred at the end of the Paleocene epoch is thought to have been caused by the release of large amounts of methane from the sea floor.

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17.2B: Nitrogen Cycle

All life requires nitrogen-compounds, e.g., proteins and nucleic acids. Air, which is 79% nitrogen gas (N_2), is the major reservoir of nitrogen. But most organisms cannot use nitrogen in this form. Plants must secure their nitrogen in "fixed" form, i.e., incorporated in compounds such as: nitrate ions (NO_3^-), ammonium ions (NH_4^+) and urea ($(NH_2)_2CO$. Animals secure their nitrogen (and all other) compounds from plants (or animals that have fed on plants).

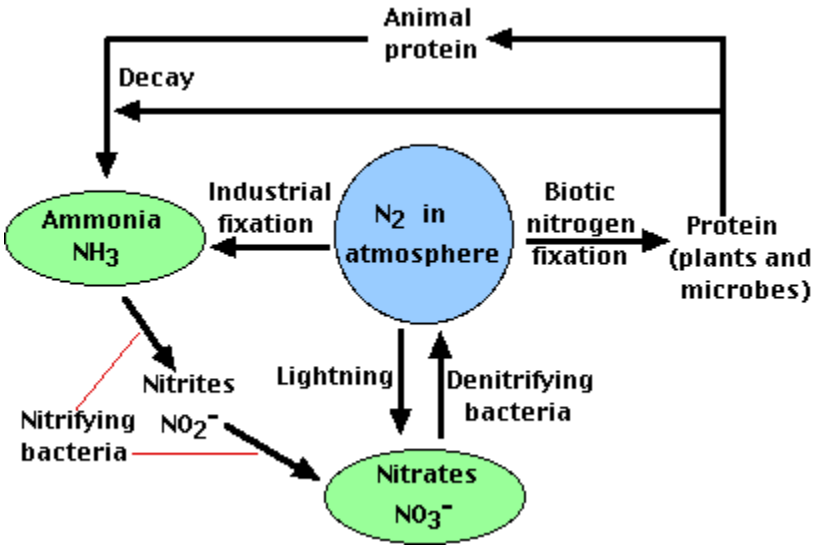


Figure 17.2.2.1 Nitrogen cycle

Four processes participate in the cycling of nitrogen through the biosphere: (1) nitrogen fixation, (2) decay, (3) nitrification, and (4) denitrification. Microorganisms play major roles in all four of these.

Nitrogen Fixation

The nitrogen molecule (N_2) is quite inert. To break it apart so that its atoms can combine with other atoms requires the input of substantial amounts of energy. Three processes are responsible for most of the nitrogen fixation in the biosphere:

- **atmospheric fixation** by lightning
- **biological fixation** by certain microbes alone or in a symbiotic relationship with some plants and animals
- **industrial fixation**

Atmospheric Fixation

The enormous energy of lightning breaks nitrogen molecules and enables their atoms to combine with oxygen in the air forming nitrogen oxides. These dissolve in rain, forming nitrates, that are carried to the earth. Atmospheric nitrogen fixation probably contributes some 5– 8% of the total nitrogen fixed.

Industrial Fixation

Under great pressure, at a temperature of 600°C, and with the use of a catalyst, atmospheric nitrogen and hydrogen (usually derived from natural gas or petroleum) can be combined to form ammonia (NH_3). Ammonia can be used directly as fertilizer, but most of its is further processed to urea and ammonium nitrate (NH_4NO_3).

Biological Fixation

The ability to fix nitrogen is found only in certain bacteria and archaea.

- Some live in a symbiotic relationship with plants of the legume family (e.g., soybeans, alfalfa).
- Some establish symbiotic relationships with plants other than legumes (e.g., alders).
- Some establish symbiotic relationships with animals, e.g., termites and "shipworms" (wood-eating bivalves).
- Some nitrogen-fixing bacteria live free in the soil.
- Nitrogen-fixing cyanobacteria are essential to maintaining the fertility of semi-aquatic environments like rice paddies.

Biological nitrogen fixation requires a complex set of enzymes and a huge expenditure of ATP. Although the first stable product of the process is ammonia, this is quickly incorporated into protein and other organic nitrogen compounds.

Decay

The proteins made by plants enter and pass through food webs just as carbohydrates do. At each trophic level, their metabolism produces organic nitrogen compounds that return to the environment, chiefly in excretions. The final beneficiaries of these materials are microorganisms of decay. They break down the molecules in excretions and dead organisms into **ammonia**.

Nitrification

Ammonia can be taken up directly by plants — usually through their roots. However, most of the ammonia produced by decay is converted into **nitrates**. Until recently this was thought always to be accomplished in two steps:

1. Bacteria of the genus *Nitrosomonas* oxidize $\mathrm{NH_3}$ to **nitrites** ($\mathrm{NO_2^-}$).
2. Bacteria of the genus *Nitrobacter* oxidize the nitrites to **nitrates** ($\mathrm{NO_3^-}$).

These two groups of autotrophic bacteria are called **nitrifying bacteria**. Through their activities (which supply them with all their energy needs), nitrogen is made available to the roots of plants. However, in 2015, two groups reported finding that bacteria in the genus *Nitrospira* were able to carry out both steps: ammonia to nitrite and nitrite to nitrate. This ability is called "comammox" (for complete ammonia oxidation).

In addition, both soil and the ocean contain **archaeal** microbes, assigned to the Crenarchaeota, that convert ammonia to nitrites. They are more abundant than the nitrifying bacteria and may turn out to play an important role in the nitrogen cycle.

Many legumes, in addition to fixing atmospheric nitrogen, also perform nitrification - converting some of their organic nitrogen to nitrites and nitrates. These reach the soil when they shed their leaves.

Denitrification

The three processes above remove nitrogen from the atmosphere and pass it through ecosystems. Denitrification reduces nitrates and nitrites to nitrogen gas, thus replenishing the atmosphere. In the process several intermediates are formed:

- nitric oxide (NO)
- nitrous oxide ($\mathrm{N_2O}$)(a greenhouse gas 300 times as potent as $\mathrm{CO_2}$)
- nitrous acid (HONO)

Once again, bacteria are the agents. They live deep in soil and in aquatic sediments where conditions are anaerobic. They use nitrates as an alternative to oxygen for the final electron acceptor in their respiration.

Anammox (anaerobic ammonia oxidation)

Under anaerobic conditions in marine and freshwater sediments, other species of bacteria are able to oxidize ammonia (with $\mathrm{NO_2^-}$) forming nitrogen gas.

$$NH_4^+ + NO_2^- \longrightarrow N_2 + 2\,H_2O \tag{17.2B.1}$$

The anammox reaction may account for as much as 50% of the denitrification occurring in the oceans. All of these processes participate in closing the nitrogen cycle.

Are the denitrifiers keeping up?

Agriculture may now be responsible for one-half of the nitrogen fixation on earth through the use of fertilizers produced by industrial fixation and the the growing of legumes like soybeans and alfalfa. This is a remarkable influence on a natural cycle. Are the denitrifiers keeping up the nitrogen cycle in balance? Probably not. Certainly, there are examples of nitrogen enrichment in ecosystems. One troubling example: the "blooms" of algae in lakes and rivers as nitrogen fertilizers leach from the soil of adjacent farms (and lawns). The accumulation of dissolved nutrients in a body of water is called **eutrophication**.

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17.2C: Symbiotic Nitrogen Fixation

Symbiotic nitrogen fixation occurs in plants that harbor nitrogen-fixing bacteria within their tissues. The best-studied example is the association between **legumes** and **bacteria** in the genus **Rhizobium**. Each of these is able to survive independently (soil nitrates must then be available to the legume), but life together is clearly beneficial to both. Only together can nitrogen fixation take place. A symbiotic relationship in which both partners benefits is called **mutualism**.

Rhizobia

Rhizobia are Gram-negative bacilli that live freely in the soil (especially where legumes have been grown). However, they cannot fix atmospheric nitrogen until they have invaded the roots of the appropriate legume.

The Infection Thread

The interaction between a particular strain of rhizobia and the "appropriate" legume is mediated by a "Nod factor" secreted by the rhizobia and transmembrane receptors on the cells of the root hairs of the legume. Different strains of rhizobia produce different Nod factors, and different legumes produce receptors of different specificity.

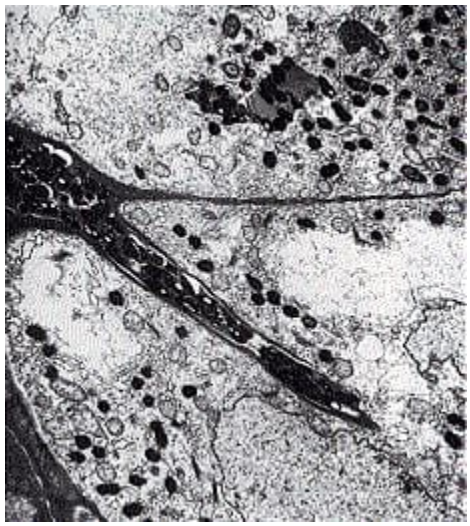


Figure 17.2.3.1 Infection thread

If the combination is correct, the bacteria enter an epithelial cell of the root; then migrate into the cortex. Their path runs within an intracellular channel that grows through one cortex cell after another. This **infection thread** is constructed by the root cells, not the bacteria, and is formed only in response to the infection. When the infection thread reaches a cell deep in the cortex, it bursts and the rhizobia are engulfed by endocytosis into membrane-enclosed **symbiosomes** within the cytoplasm. At this time the cell goes through several rounds of mitosis - without cytokinesis - so the cell becomes polyploid.

The above electron micrograph (courtesy of Dr. D. C. Jordan) shows a rhizobia-filled infection thread growing into the cell (from the upper left to the lower right). Note how the wall of the infection thread is continuous with the wall of the cell. The dark ovals are the symbiosomes.



Figure 17.2.3.2 Nitragin

The cortex cells then begin to divide rapidly forming a **nodule**. This response is driven by the translocation of cytokinins from epidermal cells to the cells of the cortex. The above photo in fig. 17.2.3.2 (courtesy of The Nitragin Co. Milwaukee, Wisconsin) shows nodules on the roots of the birdsfoot trefoil, a legume.

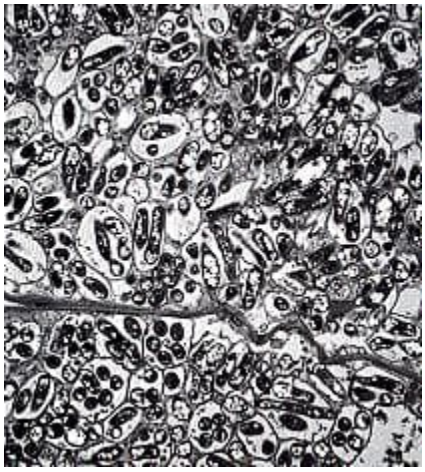


Figure 17.2.3.3 Bacteroids

The rhizobia also go through a period of rapid multiplication within the nodule cells. Then they begin to change shape and lose their motility. The **bacteroids**, as they are now called, may almost fill the cell. Only now does nitrogen fixation begin. The electron micrograph in fig. 17.2.3.3(courtesy of R. R. Hebert) shows bacteroid-filled cells from a soybean nodule. The horizontal line marks the walls between two adjacent nodule cells.

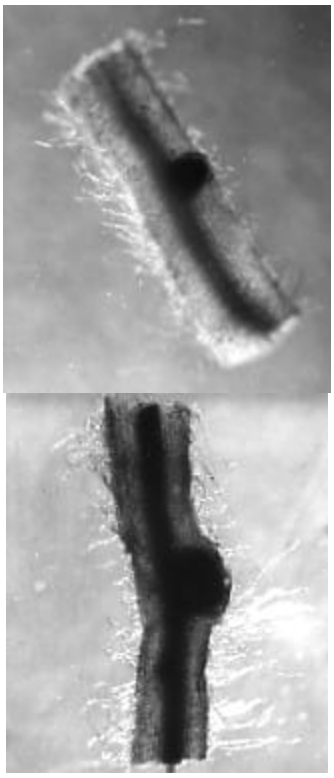


Figure 17.2.3.4 Root nodules

Root nodules are not simply structureless masses of cells. Each becomes connected by the xylem and phloem to the vascular system of the plant. The photo in fig. 17.2.3.4 on the left shows a developing lateral root on a pea root. On its right is a segment of a pea root showing a **developing nodule** 12 days after the root was infected with rhizobia. Both structures are connected to the nutrient transport system of the plant (dark area extending through the center of the root). (Photomicrographs courtesy of the late John G. Torrey.) Thus the development of nodules, while dependent on rhizobia, is a well-coordinated developmental process of the plant.

Although some soil bacteria (e.g., **Azotobacter**) can fix nitrogen by themselves, rhizobia cannot. Clearly rhizobia and legumes are mutually dependent. The benefit to the legume host is clear. The rhizobia make it independent of soil nitrogen. But why is the legume necessary? The legume is certainly helpful in that it supplies nutrients to the bacteroids with which they synthesize the large amounts of ATP needed to convert nitrogen (N_2) into ammonia (NH_3). In addition, the legume host supplies one critical component of **nitrogenase -** the key enzyme for fixing nitrogen.

The bacteroids need oxygen to make their ATP (by cellular respiration). However, nitrogenase is strongly inhibited by oxygen. Thus the bacteroids must walk a fine line between too much and too little oxygen. Their job is made easier by another contribution from their host: **hemoglobin**. Nodules are filled with hemoglobin. So much of it, in fact, that a freshly-cut nodule is red. The hemoglobin of the legume (called leghemoglobin), like the hemoglobin of vertebrates, probably supplies just the right concentration of oxygen to the bacteroids to satisfy their conflicting requirements.

The metal **molybdenum** is a critical component of **nitrogenase** and so is absolutely essential for nitrogen fixation. But the amounts required are remarkably small. One ounce (28.3 g) of molybdenum broadcast over an acre (0.4 hectare) of cropland in Australia was found to be sufficient to restore fertility for over ten years.



Figure 17.2.3.5 Molybdenum

The photo in fig. 17.2.3.5 shows that the legume clover grows normally only where the supply of molybdenum is adequate. The soil shown here (in eastern Australia) is naturally deficient in molybdenum. Although the entire fenced-in plot was seeded to clover, the plant was able to flourish and fix nitrogen only where molybdenum fertilizer had been added (foreground). (Photo courtesy of A. J. Anderson.)

Because of the specificity of the interaction between the Nod factor and the receptor on the legume, some strains of rhizobia will infect only peas, some only clover, some only alfalfa, etc. The treating of legume seeds with the proper strain of rhizobia is a routine agricultural practice. (The Nitragin Company, that supplied one of the photos above specializes in producing rhizobial strains appropriate to each leguminous crop.)

How did two such organisms ever work out such an intimate and complex living relationship? Assuming that the ancestors of the rhizobia could carry out the entire process by themselves - as many other soil bacteria still do - they must have gained some real advantage from evolving to share the duties with the legume. Perhaps the environment provided by their host, e.g., lots of food and just the right amount of oxygen, enabled the rhizobia to do the job more efficiently than before.

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17.2D: Soil

This page explains the role of soil in terrestrial food webs, highlighting its layers and the significance of topsoil for plant growth. It notes that tropical rainforest soils, although productive, tend to be nutrient-poor due to heavy rainfall and low humus levels, complicating cultivation efforts.

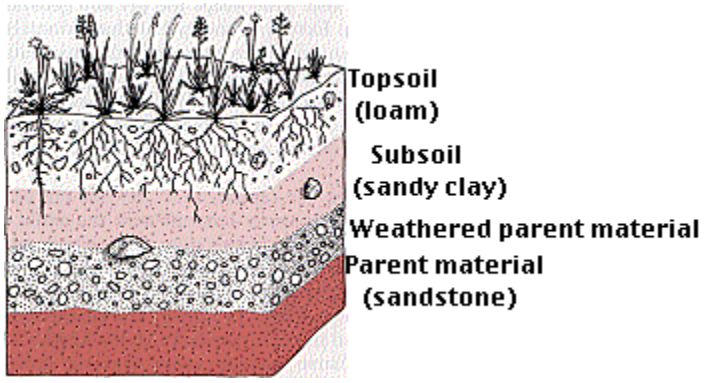


Figure 17.2.4.1 Soil profile

Topsoil

The very top layer consists of partially decayed organic debris like leaves. Beneath this is the topsoil. This horizon is usually dark in color because of **humus-** partially decayed organic matter - which has been incorporated in it from above. Humus gives the soil a loose texture that holds water and allows air to diffuse through it. Oxygen is essential to permit cellular respiration in plant roots, decay organisms, and other inhabitants of the soil.

Subsoil

The subsoil is usually lighter in color that topsoil and often contains an accumulation of **inorganic** nutrients.

Weathered parent material

This represents the first steps in the chemical breakdown of rock into soil. Often the weathered parent material is underlain by the parent material itself, although in some places it has been carried from another location by wind, water, or glaciers.

Parent material

The chemical nature of the parent material, whether granite, limestone, or sandstone, for example, has a great influence on the fertility of the soil derived from it.

The Effect of Water on Soil

The Tropical Rain Forest

The lushness of the jungle biome is somewhat illusory. While productivity is high, the soils themselves tend to be of very poor quality. Because of the high rainfall, nutrients are quickly washed out of the topsoil unless they are incorporated in the forest plants. As plant and animal debris falls to the ground, it is quickly decomposed because of the warmth and moisture there. Thus minerals are found mainly in the forest plants, not in the soil. When the plants are removed and cultivation attempted, the soils quickly lose fertility. The situation is made worse by the lack of humus (the topsoil may be no thicker than 2 in. [= 5 cm]) and the high iron and aluminum content of most of these soils. Once exposed to the sun, these lateritic soils soon bake into a bricklike material that cannot be cultivated.

The most ancient (some might say primitive) way of working these soils is still the best:

- clearing a small area of jungle
- growing crops for only a year or two, and then
- abandoning the area to jungle once again.

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17.2E: Sewage Treatment

The wastes generated by some 60% of the U.S. population are collected in sewer systems and carried along by some 14 billion gallons (~53 billion liters) of water a day. Of this enormous volume, some 10% is allowed to pass untreated into rivers, streams, and the ocean. The rest receives some form of treatment to improve the quality of the water (which makes up 99.9% of sewage) before it is released for reuse.

Biochemical Oxygen Demand (BOD)

The BOD is an important measure of water quality. It is a measure of the amount of oxygen needed (in milligrams per liter or parts per million) by bacteria and other microorganisms to oxidize the organic matter present in a water sample over a period of 5 days. The BOD of drinking water should be less than 1. That of raw sewage may run to several hundred. It is also called the "biological" oxygen demand.

Basic Sewage Treatment

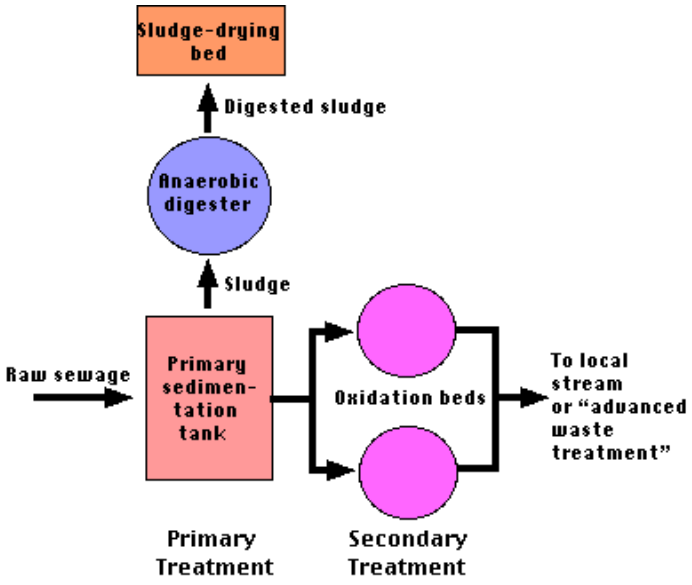


Figure 17.2.5.1 Sewage treatment

Primary Treatment

The simplest, and least effective, method of treatment is to allow the undissolved solids in raw sewage to settle out of suspension forming **sludge**. Such **primary treatment** removes only one-third of the **BOD** and virtually none of the dissolved minerals. Attempts to use digested sludge as a fertilizer have been hampered by its frequent contamination by toxic chemicals derived from industrial wastes.

Secondary Treatment

However, many treatment plants in North America then pass the effluent from primary treatment to **secondary treatment**. Here the effluent is brought in contact with oxygen and aerobic microorganisms. They break down much of the organic matter to harmless substances such as carbon dioxide. Primary and secondary treatment together can remove up to 90% of the BOD. After chlorination to remove its content of bacteria, the effluent from secondary treatment is returned to the local surface water.

Advanced Waste Treatment

The combination of primary and secondary treatment removes most of the organic matter in sewage and thus lowers the BOD. However, most of the **nitrogen** and **phosphorus** in sewage remains in the effluent from secondary treatment. These inorganic nutrients can cause eutrophication of surface water receiving the effluent causing blooms of algae. To avoid this, a few communities add a third stage of treatment called tertiary or advanced waste treatment.

Several techniques are available to remove dissolved salts from sewage effluent, but all are quite expensive.

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17.2F: Chlorination and the Law of Unintended Consequences

Chlorination is the adding of chlorine to water in order to kill any dangerous bacteria that might be present. Most municipal water supplies are chlorinated with chlorine gas, Cl_2. Swimming pools, hot tubs, and the like are usually chlorinated with chlorine-containing substances like

- calcium hypochlorite, $Ca(HClO)_2$
- sodium hypochlorite, NaHClO (bleach)
- trichloro-s-triazinetrione

In every case, the effectiveness of chlorination as a germicide is a result of chlorine's powerful oxidizing action. The widespread chlorination of municipal water supplies has been one of the public health triumphs of the past century. However, chlorine also reacts with any organic matter present in the water. Among the products formed are **chloroform** and a variety of other **trihalomethanes** (**THM**s). Although these substances are normally present only in the range of parts per billion (ppb), they nevertheless have caused considerable anxiety because several of them are known or suspected carcinogens.

The U.S. Environmental Protection Agency (EPA) sets a limit of <1 ppb of THMs in major water systems and an absolute limit of 100 ppb in any water system.

Assuming:

- that laboratory animals respond the same as humans when fed these chemicals (they may not; when **methylene chloride**, a THM, is fed to mice, it increases their incidence of cancer, but it is not carcinogenic when fed to rats),
- that we know how to scale up from doses in rats and mice to the equivalent dose in humans (there is still controversy about how best to do this),
- that there is no threshold below which doses of THMs are safe and thus
- that the concept of collective dose applies,

the EPA estimates that if everyone in the U.S. drank water containing 100 ppb of THMs for their entire lives, their chance of developing cancer (currently about 25%, representing some 500,000 cancer deaths per year) would increase by some 700 cases per year.

Turn off the chlorinators?

Claiming that they were responding to the questions raised by the U.S. EPA over the safety of THMs, officials in Peru began, in the late 80s, shutting down some of the chlorinators in the capital city, Lima, as well as in other cities and towns.

In January 1991, an outbreak of **cholera** began in several towns just north of Lima. Within weeks the epidemic of this dangerous disease (the first epidemic of cholera in the Western hemisphere in a century) spread throughout Peru and eventually through much of South and Central America. Once introduced into a city, town, or village, the disease spread rapidly through contaminated, but now unchlorinated, water supplies.

By Dec. 31, 1992 — 23 months after the epidemic began, a total of **731,312 cases** had been recorded with **6,323 deaths**. Worst hit was Peru itself. Barely 10 months into the epidemic (Nov. 13, 1991), 2,720 of its people had died of cholera. With a population of 22 million, that works out to **140** deaths per million people. Even taking the EPA's gloomiest prediction, a lifetime of drinking water containing 100 ppb of THMs would increase the rate of cancer deaths each year in Peru by less than **3** deaths per million.

What's to be done?

After the appalling devastation caused in Central and South America by misguided risk analysis, one might have hoped that the choices would be clear.

- Certainly don't suddenly stop disinfecting municipal water supplies!

- Continue to explore alternatives to chlorination.
  - For example, many water systems in France and some in the U.S. use **ozone** as the disinfectant. However, this strong oxidant also interacts with organic matter to produce undesirable contaminants.
  - Adding ammonia (NH_3) as well as chlorine to water produces **chloramine**, which is an effective disinfectant but has the disadvantage of producing carcinogenic nitrosamines and leaching lead from ancient water pipes.
  - Irradiation with ultraviolet light is least likely to produce unwanted contaminants.
- Whatever method(s) used, treat the water to reduce the amount of organic matter in it.
- Keep a cool head and try to evaluate the **size of the risks** involved before taking action.

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17.2G: Air Pollution

Human activities add a number of ingredients to the air:

- **particles**. In industrial areas and where there is heavy vehicular traffic, these contain carbon and hydrocarbons from the incomplete combustion of fuels. The hydrocarbons include a variety of polycyclic aromatic hydrocarbons (**PAH**s) that have been shown to cause mutations.

  Inhaled particles smaller than 2.5 μm ("$PM_{2.5}$") are taken deep into the lungs and may be deposited there. Chronic exposure to these small particles has been linked to

  - reduced lung development and function in adolescents;
  - an increased risk of asthma;
  - increases in the incidence of heart disease and lung cancer later in life;
  - a significant increase in the frequency of germline mutations in the sperm of exposed mice.

  The U.S. Environmental Protection Agency (EPA) has established an air quality standard of an annual average of no more than 15 μg of these particles in a cubic meter of air. (Los Angeles averaged 20 $\mu g/m^3$ in 1999 and 2000.) A survey of 217 counties in the U.S. showed an association between a reduction of 10 μg $PM_{2.5}$ with an increase in life expectancy of almost 1 year. Conversely, studies in Europe showed that an increase of 10 μg $PM_{2.5}$ was associated with a decrease in life expectancy of about 1 year.

- **sulfur dioxide** (SO_2). These are produced from the oxidation of fuels (e.g., coal and oil) containing sulfur compounds.
- **carbon monoxide** (CO). Also produced from the incomplete combustion of fuels.
- various volatile hydrocarbons including PAHs like benzopyrene, a notorious carcinogen). These are produced from the incomplete combustion of gasoline.
- **nitrogen oxides** ("**NO_x**". These are produced by the chemical union of O_2 and N_2 in the cylinders of internal combustion engines.

Photochemical smog

In bright sunlight nitrogen oxides, hydrocarbons and oxygen interact chemically to produce powerful oxidants like **ozone** (O_3) and **peroxyacetyl nitrate** (**PAN**). These **secondary pollutants** are damaging to plant life and lead to the formation of photochemical smog. PAN is primarily responsible for the eye irritation so characteristic of this type of smog. The figure outlines representative reactions leading to the formation of photochemical smog. Radicals are atoms or molecules with unpaired electrons. They are very reactive chemically. The catalytic converter in automobile exhaust systems reduces air pollution by oxidizing hydrocarbons to CO_2 and H_2O and, to a lesser extent, converting nitrogen oxides to N_2 and O_2.

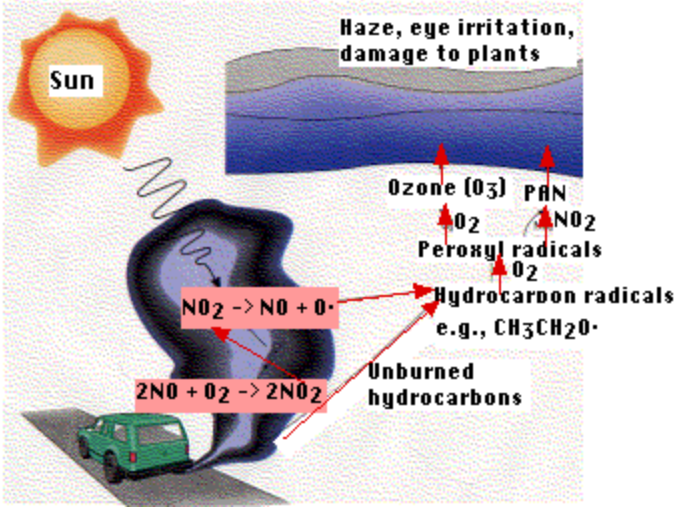


Figure 17.2.7.1 Photochemical smog

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17.2H: Acid Rain

Is rain more acid than normal. Natural rain and snow is slightly acidic (pH 5.6) because of the carbon dioxide (CO_2) dissolved in it. But over recent decades, rain in North America and Europe downwind of industrial areas has had a pH close to 4.5 and sometimes as low as 2.1 (equivalent to lemon juice).

Sulfur dioxide

The evidence is very strong that most of this acidity is caused by sulfur dioxide (SO_2) released from the smokestacks of coal-burning power plants and other industrial sources. The sulfur dioxide is converted into sulfuric acid (H_2SO_4). This may be carried to the ground in rain or snow, but often particles containing sulfuric acid settle out of dry air. So the problem of acid rain is really one of acid deposition in dry weather as well as wet.

Nitrogen oxides

Nitrogen oxides ("NO_X"), which are converted into nitric acid, also contribute to acid deposition. Automobile exhaust accounts for 50% or more of the nitrogen oxides in polluted air.

Types of damage

Acid rain has been held responsible for damaging buildings and statues made of limestone, damaging aquatic life in lakes (true), causing a decline in the vigor of U.S. and European forests (may be partially responsible), and harming human health (doubtful).

Sensitive areas

There is solid evidence that lakes in certain "sensitive" areas of North America and Europe have become more acid in recent decades. Sensitive areas are downwind of major industrial areas and where the underlying rock is granite rather than limestone. In North America, the Adirondacks of New York, the mountains of northern New England as well as large areas of southern Quebec have been particularly hard-hit. Both the plant and animal life in a lake become altered as the pH drops. The productivity of the lakes, and their content of desirable fish, decline.

The role of smokestacks

Coal burning by heavy industry was going on long before the lakes of northeastern North America began to show signs of damage. Their acidification seems to have coincided with the trend to build very tall smokestacks - often more than 500 feet (152 m) high. This was done to reduce local air pollution, but the result has been simply to transfer the problem further downwind.

Polluted air masses can cross political boundaries

Acid rain does not respect political boundaries. The lakes of Norway and Sweden suffer from the air pollution generated by the industrial areas to their south and southwest. Canadians are distressed by the damage from the air pollution generated by the industrial heartland of the U.S. The U.S. is not entirely to blame for their problems, however. Sensitive areas in Quebec are also downwind of the smelters in Sudbury, Ontario, which have the dubious distinction of generating more sulfur dioxide pollution than any other place in the entire world.

Current trends

Since the early 1980s, emissions of sulfur dioxide have been reduced in both Europe and North America. Even though nitrogen oxides have not been reduced proportionally, the result has been a reduction in the amount of acid deposition. This seems to

have stopped the acidification of lakes but not yet reversed it. The technology exists to generate electricity from coal with greatly reduced emissions and as this technology comes into use, that aspect of the problem should improve.

What about forests?

Not enough is yet known to be certain, but my guess is that sulfur dioxide will turn out to have only a supporting role to play and that the major culprit will turn out to be **ozone**. Air pollution by ozone, like that by nitrogen oxides, is largely a matter of automobile exhaust.

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17.2I: Ozone

Ozone is a highly active form of oxygen (O_3 rather than O_2). Ozone is made when a electric spark passes through air, and this accounts for the characteristic odor give off by some electrical motors. Ozone presents two quite different biological problems: too much at low levels of the atmosphere (the troposphere); too little at high altitudes (the stratosphere).

Ozone in the Troposphere

Ozone is produced by the reaction of sunlight, oxygen, and automobile exhaust (which contains hydrocarbons and nitrogen oxides). Ozone is largely responsible for the discomfort associated with **photochemical smog**. This form of smog, long familiar to people in the Los Angeles basin, is now common wherever sunlight and stagnant air occur in urban areas (Mexico City is a dramatic example with ozone levels that often exceed 100 ppb and sometimes rise above 350 ppb). High levels of ozone during smog build-up can cause difficulty to people with respiratory ailments like emphysema and asthma. Ozone also damages plants and may be an important factor in the damage that is occurring to forests in Europe and North America.

Ozone in the Stratosphere

While we often have too much ozone around us, the concentration of ozone high in the stratosphere (which begins about 7 miles [11 km] up — where airliners cruise) has declined over the past two decades. Satellite monitoring of the stratosphere, which began in 1978, has revealed a marked decline. The most serious decline occurs over Antarctica in spring (October) when a precipitous drop in ozone causes an **ozone hole**. The figure (courtesy of NASA) shows a map of the ozone hole measured over Antarctica on 5 October 1987 by a device carried on the Nimbus 7 satellite. The tips of South America (upper right quadrant) and Africa (lower right) are drawn in, as is the outline of Australia and New Zealand (lower left).

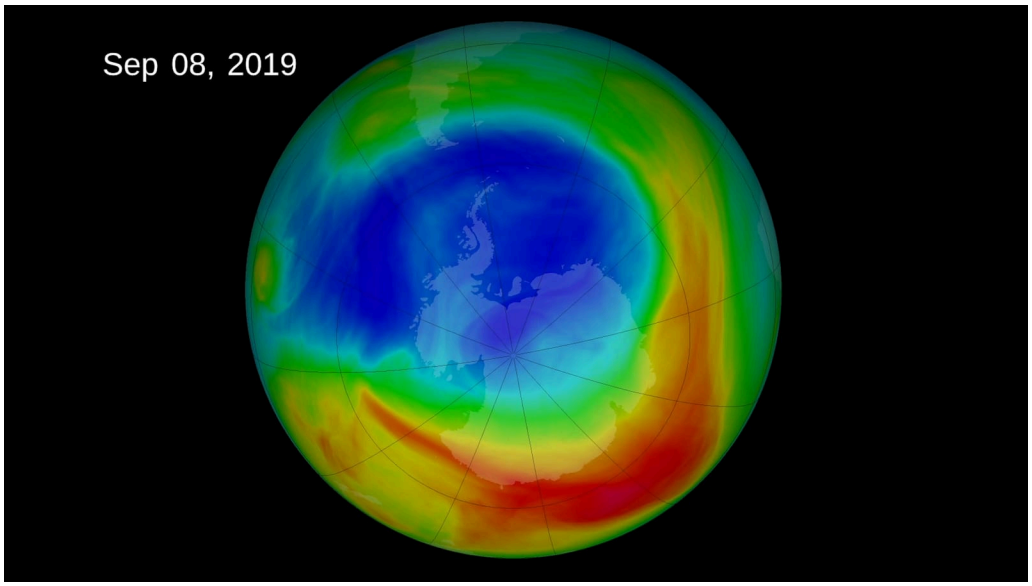


Figure $17.2I.1$: Scientists from NASA and NOAA work together to track the ozone layer throughout the year and determine when the hole reaches its annual maximum extent. This year, unusually strong weather patterns caused warm temperatures in the upper atmosphere above the South Pole region of Antarctic, which resulted in a small ozone hole. Credits: NASA Goddard/ Katy Mersmann This video can be downloaded for free at NASA's Scientific Visualization Studio

The Dobson unit is a measure of the number of molecules of ozone in a vertical column of the atmosphere. You can see that the concentration of ozone decreases in ever-smaller concentric circles with the lowest reading centered over the South Pole. Despite an anomalous increase in October 2015 (attributed to a volcanic eruption in Chile), the hole has been steadily shrinking over the last 14 years. This is probably the result of phasing out the manufacture and use of chlorofluorocarbons.

The Ozone Shield

The spreading of this ozone-depleted air may account for the more gradual and more protracted declines that are being seen at midlatitudes. From 1978-1990, average ozone levels declined 8% over Europe and about 5% over the United States. This is ominous because ozone shields the earth's surface from much of the ultraviolet radiation reaching the earth from the sun. Ultraviolet rays can cause skin cancer, cataracts, and may depress the immune system.

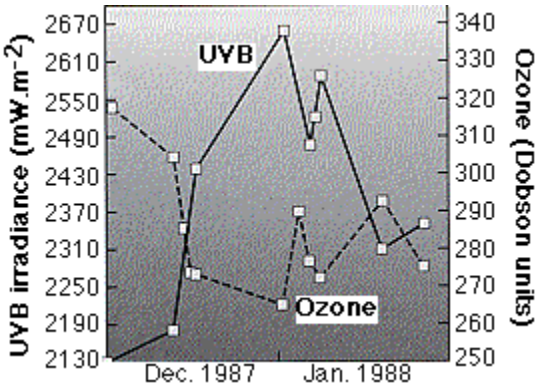


Figure 17.2I.2: O3_UVB

The graph (from C. R. Roy, et. al., in **Nature 347:**235, 1990) shows measurements of the intensity of ultraviolet light and the concentration of ozone on several sunny days in Melbourne, Australia during December 1987 and January 1988. When ozone levels were low, ultraviolet light was more intense and vice versa. The drop in ozone, which lasted about a month, was probably caused by ozone-depleted air drifting in from the ozone hole over the South Pole. Most of the ultraviolet light that reaches the earth is ultraviolet "B" (UV-B), which includes wavelengths from 280 to 320 nm.

Chlorofluorocarbons (CFCs)

Chlorofluorocarbons (CFCs) are synthetic gases in which the hydrogen atoms of methane are replaced by atoms of fluorine and chlorine (e.g., CHF_2Cl, $CFCl_3$, CF_2Cl_2). These gases are noninflammable, nontoxic, and very stable. They were widely used in industry as refrigerants (e.g., in refrigerators and air conditioners), solvents, propellants in aerosol cans (now banned in some countries), and in the manufacture of plastic foams. CFCs escape to the air from all of these uses (e.g., from leaky and discarded refrigeration units). Their chemical inertness, which makes CFCs so desirable for industry, also makes them a threat to the atmosphere. Once in the atmosphere, it may take 60–100 years for them to decompose and disappear. In the meantime, they pose a threat to the ozone shield.

Although some of the recent depletion of ozone in the stratosphere was probably due to natural causes (volcanic eruptions, fewer sunspots), some is most likely caused by chlorofluorocarbons (CFCs). However, a multi-nation agreement drawn up in 1987 - the Montreal Protocol - established a schedule for reducing the use of these materials. And, in fact, monitoring shows that the concentration of CFCs in the stratosphere has been decreasing since the mid-90s.

CFCs have largely been replaced by **hydrofluorocarbons** (HFCs) in air conditioners and refrigerators. HFCs do not destroy ozone but are potent greenhouse gases. While the ozone hole over the Antarctic still persists, there are signs of recovery of ozone levels at mid-latitudes (where we live). Whether this trend continues may depend on the contribution to ozone-depletion by nitrous oxide.

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17.3: THE GROWTH OF POPULATIONS

17.3A: The Human Population
17.3B: Principles of Population Growth

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17.3A: The Human Population

The Rate of Natural Increase (*r*)

Birth rate (*b*) – death rate (*d*) = rate of natural increase (*r*)

- birth rate expressed as number of births per 1000 per year (currently 13 in the U.S.)
- death rate expressed as the number of deaths per 1000 per year (currently 8 in the U.S.)
- So the rate of natural increase is 5 per thousand (0.005 or 0.5%).

Although the value of *r* is affected by both birth rate and death rate, the recent history of the human population has been affected more by declines in death rates than by increases in birth rates.

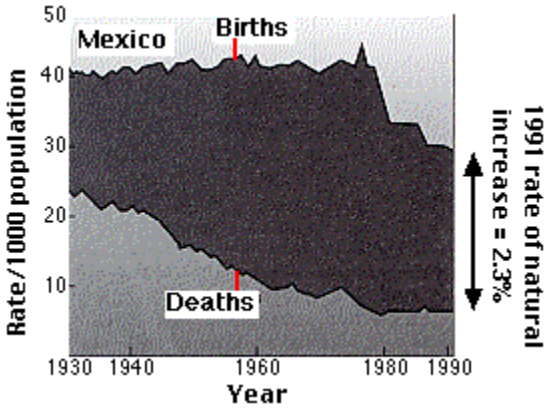


Figure 12.3.1.1 Birth & death rates in Mexico

The graph shows birth and death rates in Mexico since 1930. The introduction of public health measures, such as better nutrition, greater access to medical care, improved sanitation, and more widespread immunization has produced a rapid decline in death rates, but until recently there was no corresponding decline in birth rates. In 2012, *r* is 1.5%. (Data from the Population Reference Bureau.) Although death rates declined in all age groups, the reduction among infants and children had — and will continue to have — the greatest impact on population growth. This is because they will soon be having children of their own. This situation, resulting in a rapid rate of population growth, is characteristic of many of the poorer regions of the world.

The Demographic Transition

Slowly declining birth rates following an earlier sharp decline in death rates are today characteristic of most of the less-developed regions of the world. The shift from high birth and death rates to low birth as well as death rates is called the demographic transition.

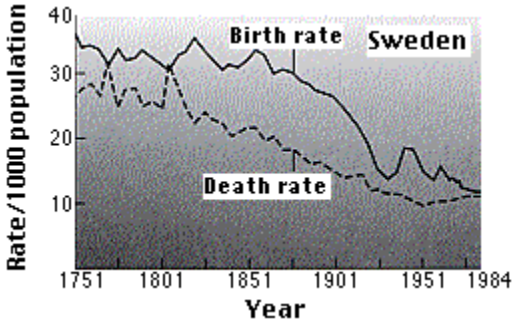


Figure 17.3.1.2 Birth & death rates in Sweden

This graph (based on data from the Population Reference Bureau) shows that the demographic transition began much earlier in Sweden than in Mexico and was, in fact, completed by the end of the nineteenth century. The spike in deaths in the interval between 1901 and 1926 was caused by the worldwide influenza pandemic of 1918–1919. The birth rate in Sweden is now (2012) 12/1000; the death rate 10/1000, giving a rate of natural increase (***r***) of 0.2%.

The Story of Sri Lanka

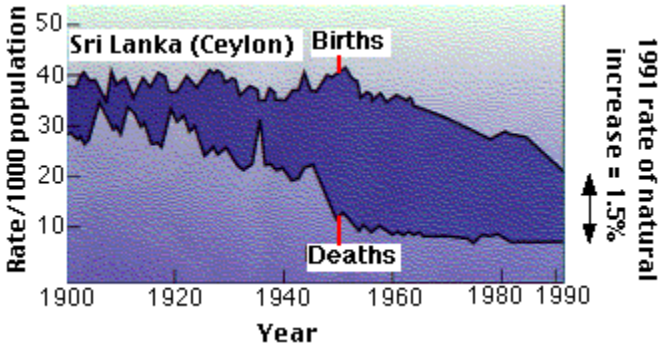


Figure 17.3.1.3 Birth & death rates in Sri Lanka

Prior to World War II, advances in public health has been largely limited to affluent, industrialized countries. But since then, improvements in public health have been made in many of the poorer countries of the world - always with dramatic effect on death rates.

- In 1945, the death rate in Sri Lanka (then called Ceylon) was 22/1000.
- In 1946, a large-scale program of mosquito control - using DDT - was started.
- By eliminating its vector, the incidence of malaria dropped sharply.
- After 9 years, the death rate dropped to 10/1000, and by 2012 was 6.
- But a compensating decline in birth rates has come more slowly (18/1000 in 2012).
- So by 2012 the population was increasing at an annual rate of 1.2% (12/1000/year).
- At this rate the population would double in 57.5 years.

Let's see why.

Exponential Growth

The prediction that Sri Lanka will double its population in 57.5 years is based on:

- the assumption that ***r*** will remain unchanged (which is surely false)
- the mathematics of exponential growth.

The product of growth grows itself. So the growth of populations is a problem in "compound interest". At the end of each year (or whatever period you choose to use), the base against which the rate is applied has grown. Whatever figures you pick, as long as ***r*** is positive, a plot of population as time elapses will produce an exponential growth curve like this one.

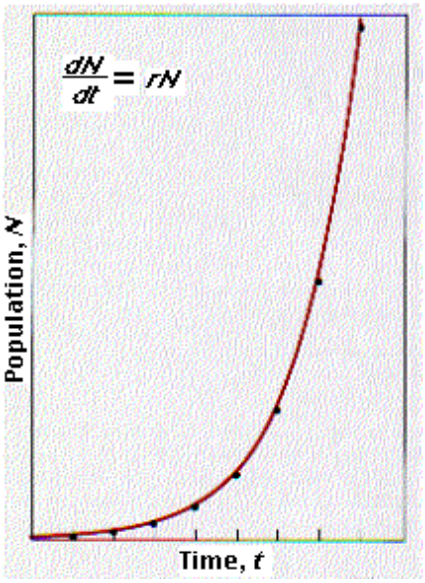


Figure 17.3.1.4 Exponential growth

The rate of population growth at any instant is given by the equation

$$\frac{dN}{dt} = rN \tag{17.3A.1}$$

where

- ***r*** is the rate of natural increase in
- ***t*** — some stated interval of time, and
- ***N*** is the number of individuals in the population at a given instant.

The algebraic solution of this differential equation is

$$N = N_0 e^{rt} \tag{17.3A.2}$$

where

- N_0 is the starting population
- ***N*** is the population after
- a certain time, ***t***, has elapsed, and
- ***e*** is the constant 2.71828... (the base of natural logarithms).

Plotting the results gives this exponential growth curve, so-called because it reflects the growth of a number raised to an exponent (***rt***).

Doubling Times

When a population has doubled,

$$N = N_0 \times 2. \tag{17.3A.3}$$

Putting this in our exponential growth equation (Equation 17.3A.2)

$$2N_0 = N_0 e^{rt} \tag{17.3A.4}$$

$e^{rt} = 2$
rt = ln (natural logarithm) of 2 = 0.69

doubling time,

$$t = \frac{0.69}{r} \tag{17.3A.5}$$

So Sri Lanka with an ***r*** of 1.2% (0.012) has a doubling time

$$t = \frac{0.69}{0.012} = 57.5. \tag{17.3A.6}$$

(You can use the same equation to calculate how quickly an investment in, for example, a certificate of deposit will enable you to double your money.)

The Population of the World

The solid line in this graph shows estimates of the size of the world's population over the last two millennia. The estimates from 1800 to 1991 are based on more accurate data than those before. The dotted line shows what would happen if exponential growth continued to the year 2100. As you can see, the world's population has been growing exponentially (except during the years of the black death). How long will it continue to do so? (Since the graph was drawn, the world's population has reached 7.1 billion; that is, in 2012 we are still on course.) But can it continue indefinitely? Surely not.

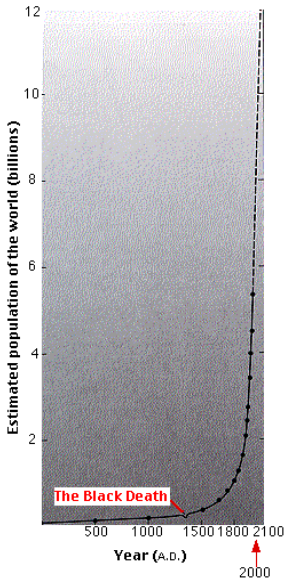


Figure 17.3.1.5 World population

Predicting Future Population Size

With a 2012 rate of natural increase in Mexico of 1.5%, its population would be expected to double in 46 years (0.69/0.015 = 46) from its 116.1 million people now to some 232 million in 2058. Will it? No one knows for certain. What actually happens to population growth depends on a number of factors. Some of these can be estimated with some confidence, some cannot. Two that can are:

- the **age structure** of the population and
- the **total fertility rate** (**TFR**).

Total Fertility Rate (TFR)

The total fertility rate is the average number of children that each woman will have during her lifetime. The TFR is an average because, of course, some women will have more, some fewer, and some no children at all. Theoretically, when the TFR = 2, each pair of parents just replaces itself. Actually it takes a TFR of 2.1 or 2.2 to replace each generation - this number is called the

replacement rate - because some children will die before they grow up to have their own two children. In countries with low life expectancies, the replacement rate is even higher (2.2–3).

Age Structure of Populations

But even a TFR of 2.1 may not ensure **zero population growth** (**ZPG**). If at one period a population has an unusually large number of children, they will — as they pass through their childbearing years — increase the *r* of the population even if their TFR goes no higher than 2.

Most childbearing is done by women between the ages of 15 and 49. So if a population has a large number of young people just entering their reproductive years, the rate of growth of that population is sure to rise.

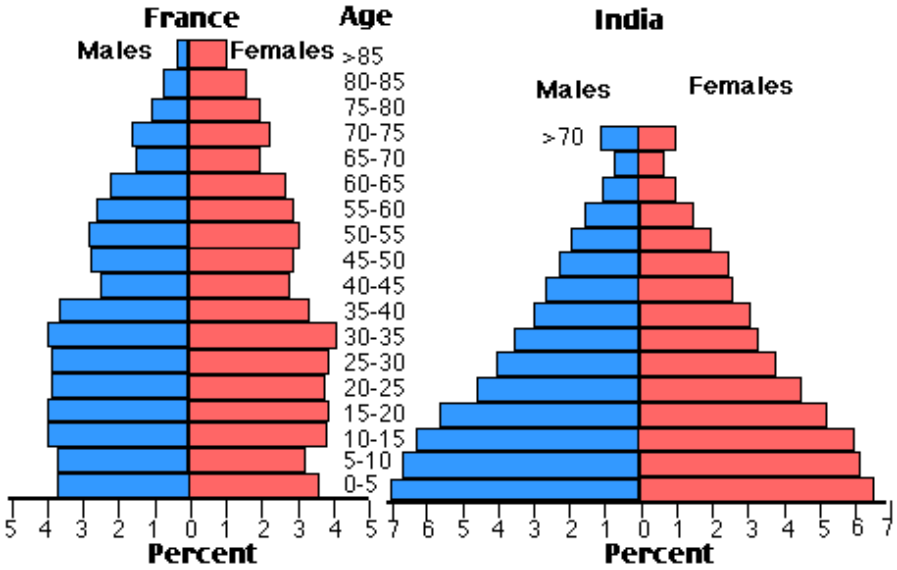


Figure 17.3.1.6 France - India population

These pyramids compare the age structure of the populations of France and India in 1984. The relative number (%) of males and females is shown in 5-year **cohorts**. Almost 20% of India's population were children — 15 years or less in age — who had yet to begin reproduction. When the members of a large cohort like this begin reproducing, they add greatly to birth rates. In France, in contrast, each cohort is about the size of the next until close to the top when old age begins to take its toll. Broad-based pyramids like India's are characteristic of populations

- with high birth rates
- low life expectancies (where many people die before reaching old age)
- advances in public health have recently reduced infant and childhood mortality

The age structure of a population also reflects the recent pattern of mortality. In countries where injuries, starvation, and disease, etc. take a heavy toll throughout life, a plot of the age cohorts produces a broad based pyramid like that of India. In France (and other countries in western Europe) almost everyone survives until old age, and a plot of the age cohorts is scarcely a pyramid at all. So even if the TFRs were the same in both countries (they are not - in India it is 2.5, in France, 2.0), India is in for more years of rapid population growth, France is not.

The U.S. Baby Boom

The TFR in the United States declined from more than 4 late in the nineteenth century to less than replacement in the early 1930s. However, when the small numbers of children born in the depression years reached adulthood, they went on a childbearing spree that produced the baby-boom generation. In 1957 more children were born in the United States than ever before (or since). These population pyramids show the baby-boom generation in 1970 and again in 1985 (green ovals).

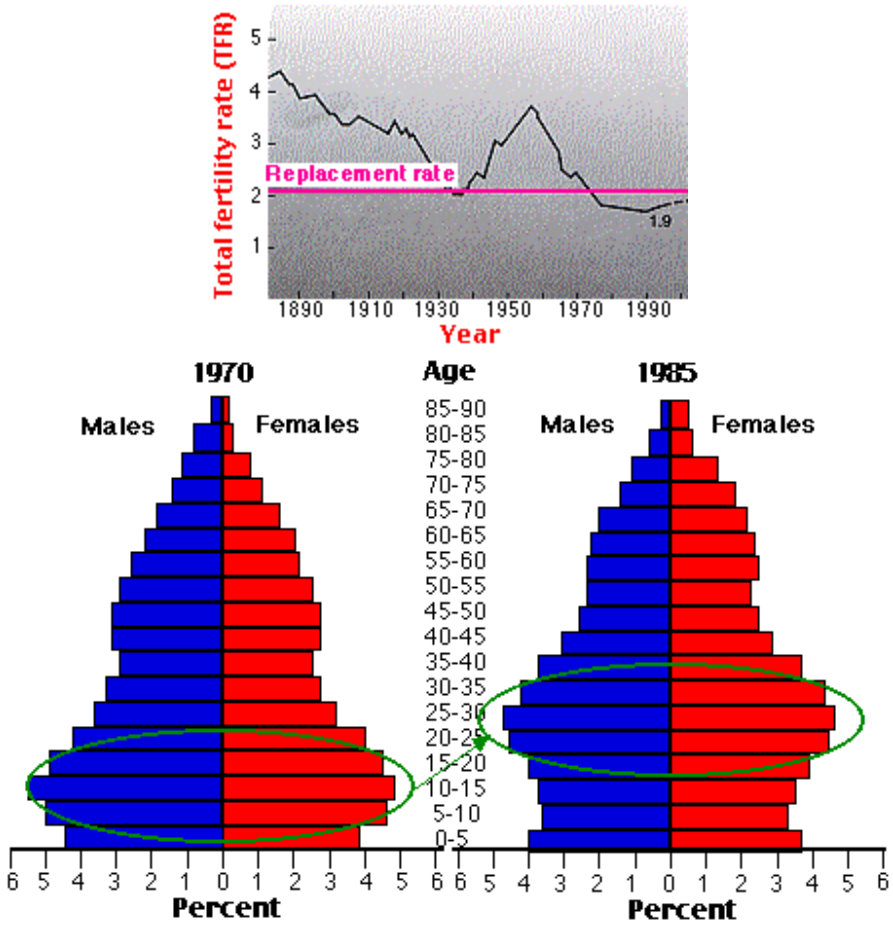


Figure 17.3.1.7 U. S Baby boom

Profound changes (e.g. enrollments in schools and colleges) have occurred — and continue to occur — in U.S. society as this bulge passes into ever-older age brackets. The baby boomers seem not to be headed for the high TFRs of their parents. They are marrying later and having smaller families than their parents. So it looks as though the **TFR** for the baby-boom generation will not exceed **replacement rate**. But this is **not** the same as zero population growth. Even with the current TFR of 1.9, this large cohort of people will keep the U.S. population growing during their reproductive years (current value for r = 0.5%).

Looking Ahead

Exponential growth cannot continue indefinitely. If the current world value for r (1.2%) remains unchanged, the world population would grow from its current 7.1 billion to 9.6 billion over the next 38 years (2050).

- Could the earth's resources sustain such a population?
- If not, how large a human population can live decently on this planet?

Some demographers (students of population) say we have already exceeded the number. Others say the earth can hold billions more.

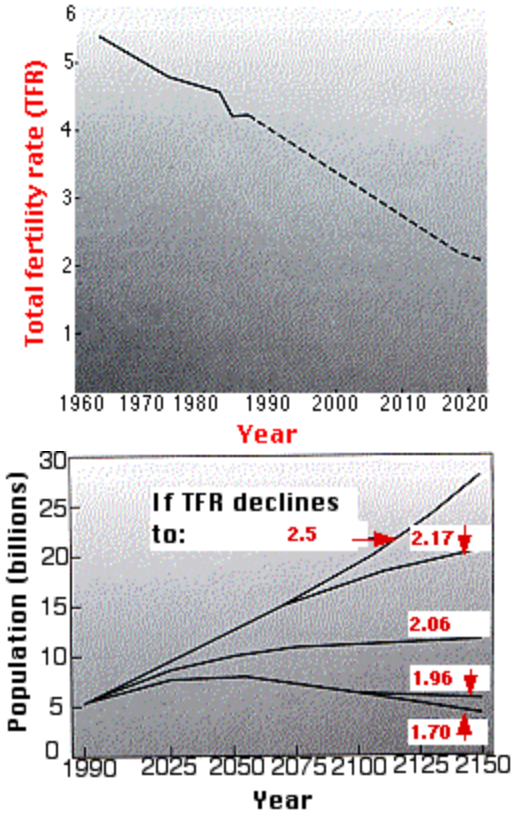


Figure 17.3.1.8 Total fertility rates

Whatever the case, there are grounds for some optimism about future population growth.

The world value for *r* peaked around 1990 and has declined since. This is a reflection of the decline in total fertility rates (TFRs) in undeveloped countries, presumably as the various factors involved in the demographic transition take hold, e.g.,

- improved standard of living
- increased confidence that your children will survive to maturity
- improved status of women
- increased use of birth control measures

The projection of future TFRs in the upper graph (from the Population Reference Bureau) predicts that the less developed countries of the world will reach replacement fertility around the year 2020. In fact, they will probably reach it sooner because by 2012 the world TFR has dropped to 2.4. Even so, will the world reach zero population growth (ZPG) then?

The right graph (based on data from the UN Long-Range World Population Projections, 1991) gives 5 estimates of the growth of the world population from now until 2150, assuming that TFRs decline from the 1991 value of 3.4 to the values shown.

- A value of 2.06 will produce a stable population of about 11.5 billion.
- A value 5% below that (1.96) will cause the population to drop back to close to 6.1 billion while
- a value of only 5% above (2.17) would produce a population of over 20 billion and still rising.

A consensus?

The several agencies that try to predict future population seem to be moving closer to a consensus that the world population will continue to grow until after the middle of this century reaching a peak of some 9.6 billion (up from the current 7.1 billion) and then perhaps declining in the waning years of this century.

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17.3B: Principles of Population Growth

Density-Independent Checks on Population Growth

The vagaries of the physical environment, for example drought, freezes, hurricane, floods, and forest fires are often check population growth. Not only may they limit population growth but they often drive existing populations well below their previous level. (And also make it unlikely that many animals will survive long enough to show signs of aging.)

These factors are described as density-independent because they exert their effect irrespective of the size of the population when the catastrophe struck.

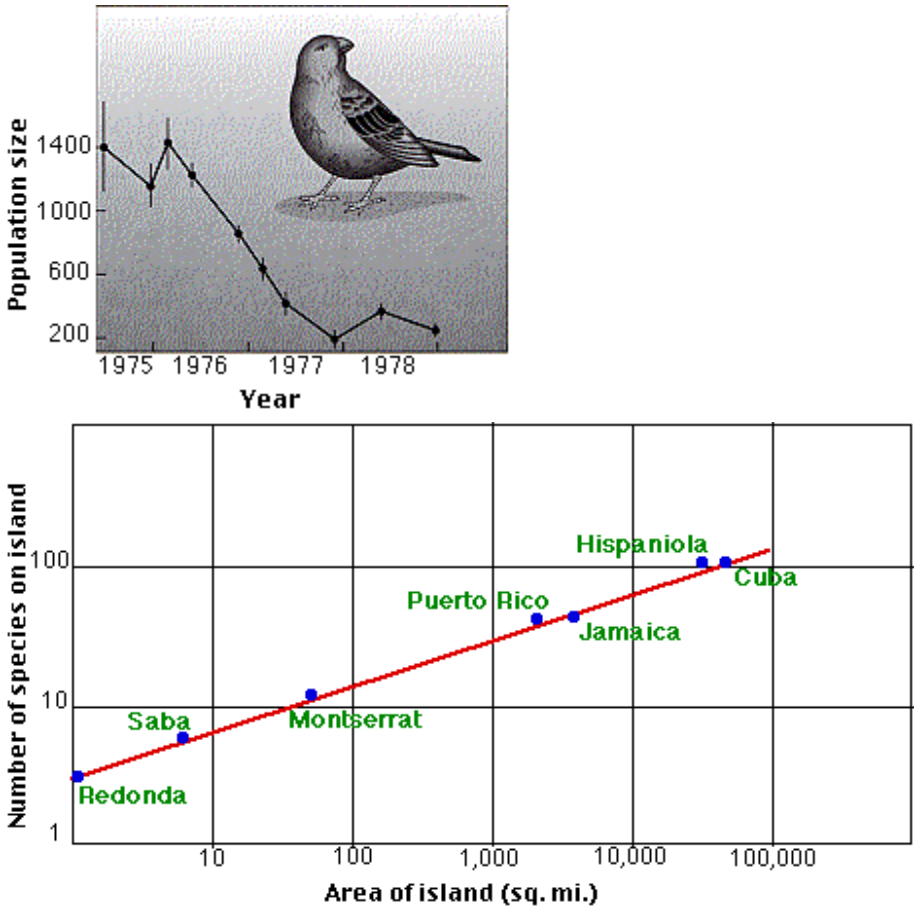


Fig.17.3.2.1 Population Density

This graph (from P. T. Boag and P.R. Grant in **Science**, **214:**82, 1981) shows the decline in the population of one of Darwin's finches (*Geospiza fortis*) on Daphne Major, a tiny (100 acres = 40 hectares) member of the Galapagos Islands. The decline (from 1400 to 200 individuals) occurred because of a severe drought that reduced the quantity of seeds on which this species feeds. The drought ended in 1978, but even with ample food once again available the finch population recovered only slowly.

Catastrophic declines are particularly risky for populations living on islands. The smaller the island, the smaller the population of each species on it, and the greater the risk that a catastrophe will so decimate the population that it becomes extinct. This appears to be one reason for the clear relationship between size of island and the number of different species it contains.

The graph (redrawn from R. H. MacArthur and E. O. Wilson, *The Theory of Island Biogeography*, Princeton University Press) shows the number of species of reptiles and amphibians on various islands in the West Indies. In general, if one island has 10 times the

area of another, it will contain approximately twice the number of species.

The same principle applies to many **habitats**. In a sense, most habitats are islands. A series of ponds, a range of mountain tops, scattered groves of citrus trees, even individual trees within a grove, all are made up of patches of habitat separated by barriers to the free migration of their inhabitants.

This has practical as well as theoretical importance. As the human population grows, jungles are cleared for agriculture, farms are paved for shopping centers, rivers are dammed for hydroelectric power and irrigation, etc. Although wildlife sanctuaries are being established, they must be made large enough so that they can support populations large enough to survive density-independent checks when they strike.

An example: Lake Guri

In 1986, the closing of a dam in Venezuela flooded over a thousand square miles (>2,500 km^2) turning hundreds of hilltops into islands. These ranged in size from less than 1 hectare (2.5 acres) to more than 150 hectares (370 acres).

Within 8 years:

- The tiniest islands (<1 hectare) lost 75% of the species that had lived there.
- The larger the island, the fewer species it lost.
- But all the islands - even the largest - lost their top predators; that is carnivores like pumas, jaguars (image), and eagles at the ends of food chains.
- Those animal species that did remain - mostly herbivores and small carnivores - greatly increased their populations because of
  - a reduction in competition for resources
  - no longer being eaten by predators

  The intense grazing by the increased herbivore populations is degrading the variety of plant life on the smaller islands.

Density-Dependent Checks on Population Growth

Intra specific Competition

Intraspecific competition is competition between members of the same species.

In the summer of 1980, much of southern New England was struck by an infestation of the gypsy moth (*Porthetria dispar*). As the summer wore on,

- the larvae (caterpillars) pupated
- the hatched adults mated
- the females laid masses of eggs (each mass containing several hundred eggs) on virtually every tree in the region
- In early May of 1981, the young caterpillars that hatched from these eggs began feeding and molting.
- The results were dramatic:
  - In 72 hours, a 50-ft beech tree or a 25-ft white pine tree would be completely defoliated.
  - Large patches of forest began to take on a winter appearance with their skeletons of bare branches.
- In fact the infestation was so heavy that many trees were completely defoliated before the caterpillars could complete their larval development.
- The result: a massive die-off of the animals; very few succeeded in completing metamorphosis.

Here, then, was a dramatic example of how competition among members of one species for a finite resource - in this case, **food -** caused a sharp drop in population.

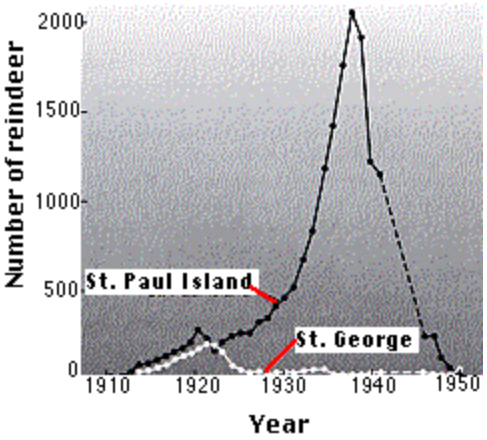


Figure 17.3.2.2 Population crash

The effect was clearly density-dependent. The lower population densities of the previous summer had permitted most of the animals to complete their life cycle. The graph shows a similar population crash; in this case of reindeer on two islands in the Bering Sea. Why the population on St. Paul Island went through so much more severe a boom-and-bust cycle than that on St. George Island is unknown. Many rodent populations (e.g., lemmings in the Arctic) go through such boom-and-bust cycles.

Inter specific Competition

All the ecological requirements of a species constitutes its **ecological niche**. The dominant requirement is usually **food**, but others, such as nesting sites and a place in the sun (for plants) may be important as well. When two species share overlapping ecological niches, they may be forced into competition for the resource(s) of that niche. This interspecific competition is another **density-dependent check** on the growth of one or both populations.

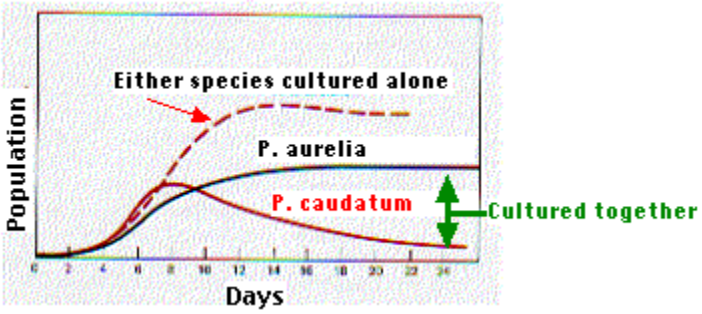


Figure 17.3.2.3 Interspecific competition

Like so many factors in ecology, interspecific competition is more easily studied in the laboratory than in the field. This graph (based on the work of G. F. Gause) shows the effect of interspecific competition on the population size of two species of paramecia, *Paramecium aurelia* and *Paramecium caudatum*.

When either species was cultured alone — with fresh food added regularly — the population grew exponentially at first and then leveled off.

However, when the two species were cultured together, *P. caudatum* proved to be the weaker competitor. After a brief phase of exponential growth, its population began to decline and ultimately it became extinct. The population of *P. aurelia* reached a plateau, but so long as *P. caudatum* remained, this was below the population density it achieved when grown alone.

The habitat of most natural populations is far more complex than a culture vessel. In a natural habitat, the species at a competitive advantage in one part of the habitat might be at a disadvantage in another. In addition, the presence of predators and parasites would limit population growth of the more successful as well as the less successful species. So, in a natural setting, the less effective competitor is usually not driven to extinction.

Character Displacement: an evolutionary response that lessens interspecific competition

When two closely-related species find themselves occupying the same geographic location (sympatric), their requirements for the necessities of life are probably so similar that they are forced into intense interspecific competition. This can lead to one of two possible outcomes.

1. The competition may be so intense that one species becomes eliminated entirely; that is, it is driven to extinction there.
2. Alternatively, the increased selection pressure may lead to **character displacement** — the evolutionary divergence of a trait they both use to exploit the resources they both depend on. Such character displacement lessens the competition between them.

Over the past several years, the research teams led by Peter and Rosemary Grant have been able to observe character displacement as it occurred between two species of Darwin's finches. This is their story.

- For many years, *G. fortis*, the **medium** ground finch, lived on Daphne Major without its relatives, *G. fulginosa*, the **small** ground finch, and *G. magnirostris*, the **large** one.
- However, in 1982, *G. magnirostris* arrived and over the following years grew in population.
- These were good years, with plenty of food, and the two species coexisted nicely.
- However, a severe drought in 2003–2004, sharply reduced the available food including the large seeds that magnirostris and the larger-beaked members of the fortis population competed intensely for.
- The result was a massive die-off of both species with the larger-beaked members of the fortis population suffering greater mortality than the smaller-beaked members of the species.
- The offspring of the fortis survivors, born in 2005, had beaks that averaged over 5% smaller than the average of the parental population. (After the drought of 1977, when *G. magnirostris* was not on the island to compete with fortis, their beak size increased rather than decreased.)
- This study of character displacement is described in the 14 July 2006 issue of **Science**.

Reproductive Competition

Declining birth rates also lead to reduced population growth.

We know that humans make deliberate family planning choices, but analogous behavior is found in other animals as well.

- Fruit flies living under crowded conditions lay fewer eggs.
- Laboratory rats in a confined area soon reach a stable population size even though abundant food is available. The main cause is a sharp rise in infant mortality. Reduced maternal care and even cannibalism take a heavy toll of the newborn.
- The honeybee queen regulates her rate of egg laying to the availability of food: reducing it during periods of poor flowering and ceasing entirely in the late summer.

An alternative to limiting the number of offspring per pair of parents is to limit the number of parents. Some mammals and birds achieve this by establishing breeding territories. Each mating pair occupies an area of a size sufficient to supply its needs including those of its offspring. One or both members defend this territory against intrusion from other members of the **same** species. This behavior not only ensures that the resources on which they depend will not be exceeded but may keep the population in check by preventing breeding among its surplus members.

Social conventions among humans (e.g., attitudes about the proper age of marriage and desirable family size) also have a marked influence on birth rates. However social conventions - and the birth control techniques that may supplement them — have been most successful at reducing birth rates among just those people least in need of it. In the poorer countries, early marriage, a desire for large families, and failure to employ birth control methods reliably are common.

Migration

Migration is often an important density-dependent factor in reducing populations. As the population increases, many of its members emigrate.

Predation

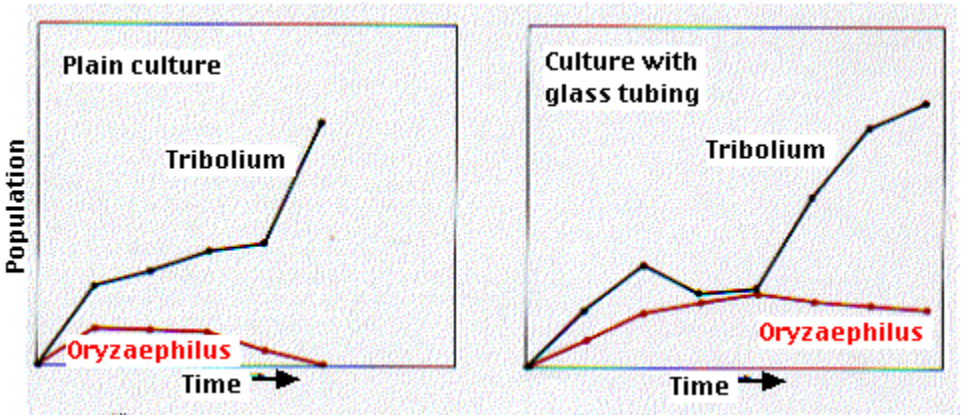


Figure 17.3.2.4 Population changes

As a population increases, its predators are able to harvest it more easily. These graphs (based on data from Crombie, A. C., *Journal of Animal Ecology*, **16:**44, 1947) show the population changes among flour beetles grown in plain flour (left) and in flour containing pieces of glass tubing.

Each culture was started with four adults of each species. In plain medium, after an initial spurt of both populations, Tribolium continued to expand its numbers while the Oryzaephilus population declined and was eventually driven to extinction (left). Several factors were at work, but predation was by far the most important.

- Tribolium adults feed voraciously on the eggs and pupae of Oryzaephilus.
- But Oryzaephilus adults do not feed so vigorously on Tribolium eggs and do not eat their larvae at all.

Glass tubing provided a refuge for some Oryzaephilus larvae enabling them to complete their life cycle. This reduction in the intensity of predation permitted the two populations to coexist indefinitely (right).

Parasitism

Parasites are able to pass from host to host more easily as the population density of the host increases. For this reason, epidemics among humans are particularly severe in cities. In fact, for most of the period since humans began living in cities, city populations have been maintained only through continual immigration from the countryside. Not until the development of community sanitation, immunization, and other public health measures did cities avoid periodic sharp drops in population as a result of epidemics.

The recurrent epidemics of the "black death" in Europe that began in the fourteenth century caused a sharp decline in population. In just 3 years (1348–1350), at least one-quarter of the population of Europe died from the disease (probably plague). More recently, the great influenza pandemic of 1918–1919 is thought to have killed over 20 million people worldwide.

The house finch, *Carpodacus mexicanus*, — native to western North America — is a recent immigrant to the eastern United States where it is parasitized by a mycoplasma that reduces the lifespan and fecundity of the birds. Data collected by amateur bird watchers show that the arrival of the disease (in the mid-90s) in areas with a high population of the birds drove their numbers down more than it did in regions of low finch populations. Whatever the starting value, all infected populations ended up with similar populations. This is a clear example of the **density-dependent** effect of parasitism on a population.

Population Cycles

Some populations go through repeated and regular periods of boom followed by bust.

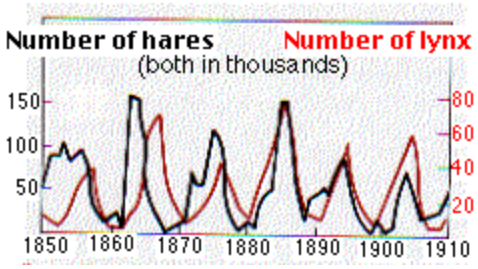


Figure 17.3.2.5 Population fluctuation

This graph shows the 10-year cyclical fluctuations in the populations (measured by counting the hides offered for sale at the Hudson Bay trading posts in Canada) of the varying hare ("snowshoe rabbit") and its chief predator, the lynx, from 1850 to 1910. The size of the lynx population was closely dependent on the size of its prey (hare) population. The factors causing the hare population to go through its boom-and-bust cycles are still debated, but predation by lynxes was probably only one factor.

Recent field studies have provided clearer answers for three other cyclical populations, voles (a small rodent) in Finland, the red grouse in Scotland, and lemmings (another small rodent) in Greenland.

Voles

The vole population in Finland regularly goes through 3-year cycles of boom-and-bust. When Korpimäki and Norrdahl removed all their predators (both mammals and birds) from their test areas, the cycles ceased. Here, then, the cycles were driven by the density-dependent check of **predation**.

Red grouse

The red grouse population in Scotland goes through cycles of 4–8 years. From peak to trough, the population may decline by a factor of 1000. These cycles do not appear to be caused by the hunting of this popular game bird.

The birds are parasitized by a nematode, and infected birds have lower fecundity (birth rates down) and higher mortality (death rates up) than uninfected birds.

P. J. Hudson and his colleagues treated large numbers of birds in several test areas with a drug to prevent or cure an infection. The populations in the treatment areas ceased to cycle. It was not necessary to treat all the birds; 20% of them seem enough to prevent epidemics (just as immunization of humans doesn't have to reach 100% to put an end to pathogen transmission).

Here, then, the cycles were driven by the density-dependent check of **parasitism**.

Lemmings

A 15-year study of the population of lemmings in northeast Greenland was reported by Gilg, O., *et al.*, in **Science**, 31 October 2003. These workers showed that the lemming population rises and falls with a cycle of 4 years. The population of the shorttail weasel (aka ermine, stoat), the principal predator of the lemming, does as well but with a 1-year lag behind the lemming population.

Because of this lag, one might expect that the lemming population would continue to outstrip the weasel population until the lemmings bumped into the carrying capacity of their environment (e.g., availability of food and nesting sites). But this does not occur because as the lemming population grows, other predators (e.g., foxes and owls) shift their diet in favor of lemmings.

As the lemming population then begins to decline,

- these flexible predators return to their former food sources while
- the more "picky" weasels decline in numbers as their sole food source, the lemmings, have.

The Carrying Capacity of the Environment (*K*)

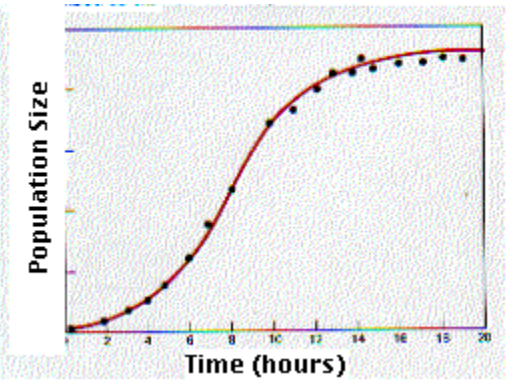


Figure 17.3.2.6 Yeast growth

This graph shows the growth of a yeast population in culture. After a period of exponential growth, the size of the population begins to level off and soon reaches a stable value. This type of growth curve is called sigmoid or **S-shaped**. If we add fresh culture medium to the container, exponential growth resumes until a new, higher plateau is reached. Evidently the growth rate (***r***) declines as the density of the population approaches a certain limiting value.

When ***r*** = 0, ***dN/dt*** = 0 and the population ceases to grow. The yeasts have reached **zero population growth** or **ZPG**.

The causes:

- running out of food
- accumulation of ethanol. (When its concentration reaches 12–14%, the yeast die (which explains the maximum alcohol content of natural alcoholic beverages like wine).

The limiting value of the population that can be supported in a particular environment is called its **carrying capacity** and is designated ***K***. When the population is far below ***K***, its growth is exponential, but as the population approaches ***K***, it begins to encounter ever-stronger "**environmental resistance**". Let us use the expression

$$\frac{K - N}{K}$$

as a "growth realization factor", that is, a factor representing the degree to which the population can actually realize its maximum possible rate of increase. Introducing this factor into our original (exponential) growth equation, we get

$\frac{dN}{dt} = rN$	(	$\frac{K - N}{K}$	)

The equation tells us that

- If the size of the population (N) is far below the carrying capacity of the environment (K), the growth realization factor will be close to 1, and the population will show exponential growth.
- But as N begins to approach K, the growth realization factor approaches zero, and the rate of population growth drops to zero:

$\frac{dN}{dt} = 0$ = "**ZPG**" (zero population growth)

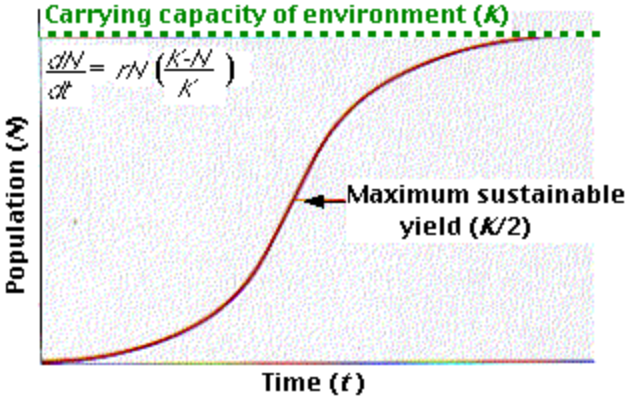


Figure 17.3.2.7 Logistic curve

Plotting the growth of a population from an initial growth realization factor of 1 to a final factor of 0 produces a curve like this, called the **logistic growth curve** or **S-shaped** curve of growth. Although actual populations are unlikely to follow the theoretical logistic growth curve exactly, the curve can provide us with valuable guidance in managing populations.

Example 1: The logistic curve tells you that you are unlikely to rid your house of a large rat population by setting rat traps. No matter how many you put out, the *r* for rats is so high (perhaps 0.0147 per day) that they will reproduce faster than you can catch them. What you must do instead is to prevent them from getting food in and around your house. With a sharply-reduced K, their population will decline.

Example 2: The converse of the pest problem is how to keep endangered species from becoming extinct. But outlawing hunting will have no appreciable impact if the habitat on which that species depends for its K — pasture or woods or whatever — disappears under the parking lot of a shopping plaza.

Example 3: Modern intensive fishing methods have repeatedly produced ominous declines in the catch of many species as the populations have been unable to maintain themselves. The logistic curve provides a goal to managing fisheries: harvest at only such a rate that the population is maintained at *K*/2. At this size, the population is able to grow most rapidly. The value *K*/2 is known as the **maximum sustainable yield**.

r -Strategists and *K* -Strategists

r-strategists

I once plowed up an old field and allowed it to lie fallow. In the first season it grew a large crop of ragweed.

Ragweed is well-adapted to exploiting its environment in a hurry - before competitors can become established. It grows rapidly and produces a huge number of seeds (after releasing its pollen, the bane of many hay fever sufferers). Because ragweed's approach to continued survival is through rapid reproduction, i.e., a high value of *r*, it is called an *r*-strategist. Other weeds, many insects, and many rodents are also *r*-strategists. If fact, if we consider an organism a pest, it is probably an *r*-strategist.

In general *r*-strategists share a number of features:

1. They are usually found in disturbed and/or transitory habitats. In the second season of my field, perennial grasses and wildflowers had produced a dense carpet of mixed vegetation and not a ragweed plant was to be found.
2. They have short life spans. The house mouse, with a maximum life span of 3 years, is an *r*-strategist.
3. They begin breeding early in life.
4. They usually have short generation times; that is, they have short gestation periods and are soon ready to produce another crop of young. The housefly can produce 7 generations each year (each of about 120 young).
5. They produce large numbers of offspring. The American oyster, releasing a million eggs in one season, is an *r*-strategist. Most of its offspring will die, but the sheer size of its output increases the likelihood that some offspring will disperse to new habitats.
6. They take little care of their offspring, and infant mortality is huge. If we plot a survivorship curve for an *r*-strategist, it is apt to take the form of the curve labeled **D**. Although humans are not *r*-strategists, the higher birth rate in some countries may

well be a response to their higher rates of infant mortality (curve **B**).
7. They have efficient means of dispersal to new habitats.

For *r*-strategists, alleles that enhance any of the traits listed above will be favored by natural selection. Hence, *r*-strategists are said to be the product of ***r*-selection**.

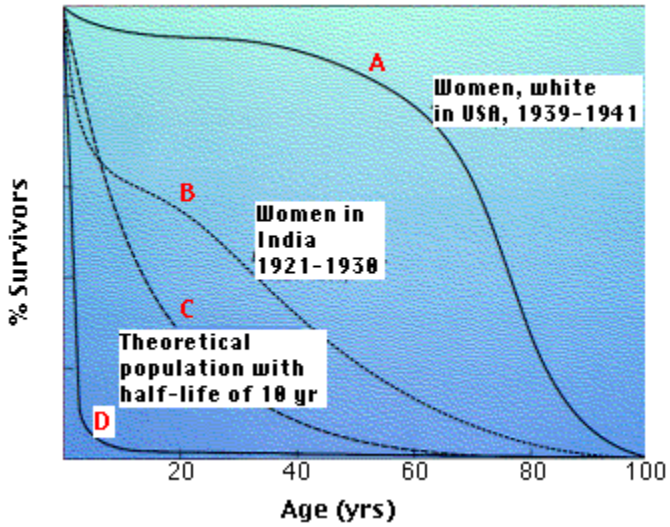


Figure 17.3.2.8 Survivorship curves

The graph shows 4 representative survivorship curves. The vertical axis gives the fraction of survivors at each age.

- Curve **A** is characteristic of organisms that have low mortality until late in life when aging takes its toll.
- Cure **B** is typical of populations in which such factors as starvation and disease obscure the effects of aging, and infant mortality is high.
- Curve **C** is a theoretical curve for organisms for which the chance of death is equal at all ages. This might be the case for organisms that do not age (some fishes) or those that suffer severe random mortality throughout life (e.g., many songbirds). *K*-strategists usually have survivorship curves somewhere between **A** and **C**.
- Curve **D** is typical of organisms, oysters for example, that produce huge numbers of offspring accompanied by high rates of infant mortality. Many *r*-strategists have such a curve.

K-strategists

When a habitat becomes filled with a diverse collection of creatures competing with one another for the necessities of life, the advantage shifts to *K*-strategists. *K*-strategists have stable populations that are close to *K*. There is nothing to be gained from a high *r*. The species will benefit most by a close adaptation to the conditions of its environment.

Typically, *K*-strategists share these qualities:

1. They are usually found in stable habitats. Most of the species in a mature forest will be *K*-strategists.
2. They have long life spans. The elephant and the tortoise are *K*-strategists.
3. They begin breeding later in life.
4. They usually have long generation times. It takes 9 months to produce a human baby.
5. Most produce small numbers of offspring. Birds are *K*-strategists, most species producing fewer than a dozen young each year.
6. They take good care of their young. Infant mortality tends to be low. If we plot a survivorship curve for a *K*-strategist, it usually lies somewhere between curve **A** (above), where most of the population dies of old age, and curve **C**, where all ages are equally at risk of being struck down by random hazards.
7. *K*-strategists typically have evolved in such a way that they become increasingly efficient at exploiting an ever-narrower slice of their environment. Thus it is not surprising that many endangered species are *K*-strategists.

For *K*-strategists, alleles that enhance their ability to exploit the resources of their habitat; that is, to increase the carrying capacity, *K*, of their environment, will be favored by natural selection. Hence, *K*-strategists are said to be the product of

***K*-selection**.

Population density can cause shifts in strategy

A team at the Santa Cruz campus of the University of California (Sinervo et al., in the 21 August 2001 issue of **Nature**) studied the boom-and-bust cycles of the native side-blotched lizard. They found that the lizard population went through 2-year cycles of boom and bust.

Year 1 = Boom

- A low population of adults
- living well below the carrying capacity (*K*) of their environment
- produced large numbers of young (an *r*-strategy)
- leading to rapid overcrowding and

Year 2 = Bust

- A large population of adults
- living close to or above the *K* of their environment
- produced fewer surviving young
- leading to a sharp decline in population and

Year 3 = another Boom year, and so on.

They also found that the population is polymorphic containing:

- females with **orange throats** that produced as many as 5 clutches of eggs (averaging 6 eggs per clutch) a season. It takes lots of food reserves to make eggs and the eggs of these highly-prolific orange-throated females tended to be smaller — and to hatch into smaller lizards — than those of the
- females with **yellow throats**. These females tend to lay fewer, but larger, eggs, and the young lizards that hatch from them are larger than those produced by orange-throated mothers.

As they predicted, it turned out that:

- **Orange**-throated lizards are ***r*-strategists**. In boom years, they were more successful than the yellow. The population explosion of young lizards produced by them led to next year's bust.
- **Yellow**-throated lizards are ***K*-strategists**. Producing smaller numbers of larger lizards, they were more successful at leaving surviving offspring to lay the groundwork for the next boom year.

Here, then, intraspecific competition has created a population cycle alternately favoring *r*-strategists and *K*-strategists.

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17.4: INTERACTIONS BETWEEN SPECIES



- Topic hierarchy

17.4A: Symbiosis

17.4B: Insecticides

17.4C: Biological Control of Pests

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17.4A: Symbiosis

Most of the interactions between species involve food, i.e., competing for the same food supply, eating (predation) and avoiding being eaten (avoiding predation). These interactions are often brief. There are many cases, however, where two species live in close association for long periods. Such associations are called **symbiotic** ("living together"). In symbiosis, at least one member of the pair benefits from the relationship. The other member may be injured (**parasitism**), relatively unaffected (**commensalism**) or may also benefit (**mutualism**). (Some people restrict the term symbiosis to only these mutually beneficial interactions, but we shall not.)

Mutualism

Symbiotic relationships in which **each species benefits** are mutualistic. There are hundreds of examples of mutualism between a heterotroph and an alga.

- *Paramecium bursaria* is a ciliate that engulfs unicellular green algae into vacuoles within its cell.
  - The paramecium certainly benefits from the food synthesized by the alga. It can be cultured apart from the alga but then must be given extra food.
  - The alga presumably benefits from the carbon dioxide produced by its host as well as the host's ability to transport it to a spot where there is ample light.
- Many other aquatic heterotrophs
  - sponges
  - sea anemones
  - planarians
  - clams

  also harbor algae within their cells.

Mutualistic relations between plants and fungi are very common. The fungus invades and lives in or among the cortex cells of the secondary roots. The association is called a **mycorrhiza**. The fungus helps the host plant absorb inorganic nitrogen and phosphorus from the soil. Some mycorrhizal fungi also secrete antibiotics which may help protect their host from invasion by parasitic fungi and bacteria. Many mushrooms are the spore-forming bodies of mycorrhizal fungi. The truffle is often found in oak forests because the fungus that produces it establishes mycorrhiza on oak roots.

Endosymbiosis

Endosymbiosis is a **mutualistic** relationship between a host and an organism living within its body or cells.

The pea aphid and its endosymbiont

The pea aphid, *Acyrthosiphon pisum*, is an insect pest that sucks the juices from its host plant. However, plant sap is deficient in several essential amino acids. The pea aphid thrives nonetheless thanks to specialized cells within its body that contain the gamma proteobacterium, *Buchnera aphidicola*, that can live nowhere else. The genome of this obligate intracellular Gram-negative bacterium encodes a number of enzymes needed to complete the synthesis of the amino acids needed by its host. In return, the aphid's genome encodes enzymes needed by *Buchnera* to synthesize its lipopolysaccharide cell wall and has lost genes that might otherwise repel infection by Gram-negative bacteria.

Symbiotic nitrogen fixation

One of the most important examples of mutualism in the overall economy of the biosphere is the endosymbiotic relationship between certain nitrogen-fixing bacteria and their legume hosts. A large body of evidence supports the view that intracellular

endosymbiotic relationships gave rise to eukaryotes with their mitochondria and chloroplasts.

Cleaning Symbiosis

The drawing shows the Nile crocodile opening its mouth to permit the Egyptian plover to feed on any leeches attached to its gums. Cleaning symbiosis is more common in fish.

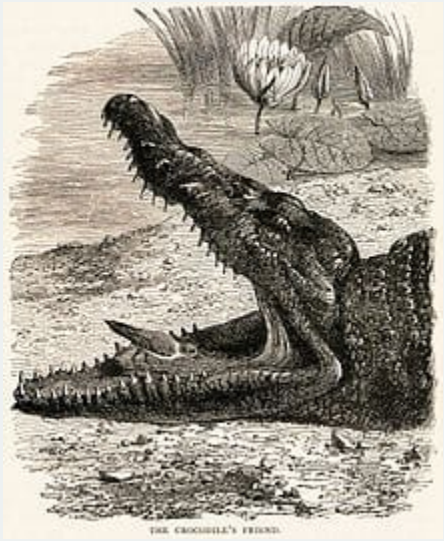


Figure 17.4.1.1 Cleaning symbiosis. Herodotus asserted that the trochilus bird, possibly a sandpiper, was able to enter the mouth of the Nile crocodile in what would now be called a cleaning symbiosis. Drawing by Henry Scherren, 1906

Commensalism

Commensalism means "at table together". It is used for symbiotic relationships in which one organism consumes the unused food of another. Some examples:

- the remora and the shark. The dorsal fin of the remora (a bony fish) is modified into a sucker with which it forms a temporary attachment to the shark. When the shark feeds, the remora picks up scraps. The shark makes no attempt to prey on the remora.
- Some species of barnacles are found only as commensals on the jaws of whales. And there are other species of barnacles found only as commensals on those barnacles!
- Many of the bacteria living in our large intestine. They feed on food which we cannot digest and do not harm us. And some help us; that is, the relationship is mutualistic. Animals (e.g., mice) raised under germfree conditions are abnormal in several ways, e.g.,
  - they have elevated levels of a subset of NKT cells
  - reduced levels of regulatory T cells (Treg) - both effects predisposing the animals to
  - asthma and inflammation of the intestine

  So it is now standard practice to deliberately infect them with several species of microorganisms so that the animals develop normally.

Epiphytes

Epiphytes are plants that live perched on sturdier plants. They do not take any nourishment from their host and simply benefit from being better exposed to sunlight. Some examples include many orchids and many bromeliads (e.g., "Spanish moss" and other members of the pineapple family).

Parasitism

A parasite is an organism that lives on or in the body of another organism (the host) from whose tissues it gets its nourishment,

and to whom it does some damage. Animals are parasitized by viruses, bacteria, fungi, protozoans, flatworms (tapeworms and flukes), nematodes, insects (fleas, lice), and arachnids (mites). Plants are parasitized by viruses, bacteria, fungi, nematodes, and a few other plants. Parasites damage their host in two major ways:

- consuming its tissues, e.g., hookworms
- liberating toxins, for example,
  - Tetanus bacilli secrete tetanus toxin which interferes with synaptic transmission.
  - Diphtheria bacilli secrete a toxin that inhibits protein synthesis by ribosomes.

The relationship between parasite and host varies along a spectrum that extends from "hit and run" parasites that live in their host for a brief period and then move on to another with or without killing the first to parasites that establish chronic infections. Both parasite and host must evolve to ensure the survival of both because if the parasite kills its host before it can move on, it destroys its own meal ticket

Rabbits in Australia

In 1859, the European rabbit was introduced into Australia for sport. With no important predator there, it multiplied explosively. The raising of sheep (another imported species) suffered badly as the rabbits competed with them for forage. This picture (courtesy of Dunston from *Black Star*) gives you the idea. Having removed all forage plants, which ordinarily supply them with water as well as food, the rabbits had to drink from a pool.



Figure 17.4.1.2 Australian rabbits. The mutual evolutionary adaptations of parasite and host may lead to the parasite becoming less damaging at the same time as the host becomes more resistant.

In 1950, the **myxoma virus** was brought from Brazil and released. The epidemic that followed killed off millions of rabbits (perhaps 99.5% of the population). Green grass returned and sheep raising once again became profitable. But the rabbits were not eliminated. In fact, although small epidemics still occur, the rabbit population has recovered somewhat (although nowhere near its pre-1950 levels). What happened? Thanks to careful planning, we know.

- The rabbits today are more resistant to infection than their predecessors. This can be measured by infecting them with the original strain that has been maintained in the laboratory.
- At the same time, the virus circulating in the wild rabbits has become less virulent. This can be measured by determining the % mortality of laboratory rabbits when they are infected with the current strain of virus.

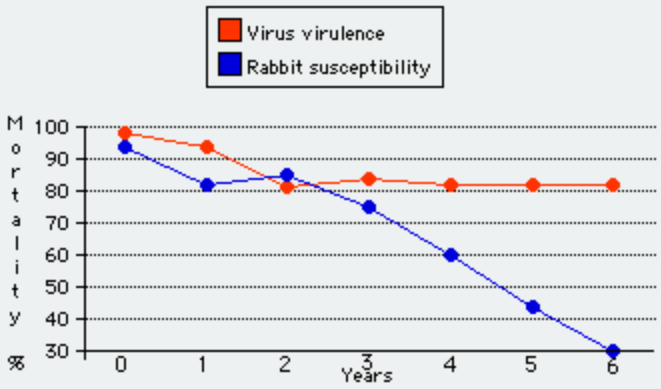


Figure 17.4.1.3 Evolutionary adaptations

The graph (based on data of Sir Macfarlane Burnet and D. O. White) shows these mutual evolutionary adaptations over the first six years after the introduction of the virus.

The "Degeneracy" of Parasites

During the course of adapting to conditions in their host, parasites often lose structures and functions that were essential for their ancestors (and any free-living relatives). The tapeworm has no eyes, no digestive tract, and only vestiges of nervous, excretory, and muscular systems. While you may call them degenerate, these losses represent a gain in efficiency and improved specialization. What good would these structures be anyway in the human intestine? On the other hand, the tapeworm produces hundreds of proglottids: egg-forming machines that improve the likelihood that the tapeworm will leave descendants that reach another host.

This emphasis on reproduction is also seen in

- *Rafflesia*, a parasitic angiosperm found in Malaysia. It has no roots, stems, or leaves, although it does have tubes which penetrate the tissues of its host. But it has a huge flower (~3 feet or 1 meter in diameter).
- *Sacculina*, a barnacle that parasites crabs. The adult consists of little more than a sac (hence the name) containing reproductive organs. Not until its larvae were discovered could it even be determined that Sacculina was a crustacean.

Mycobacterium leprae: pseudogenes

M. leprae causes leprosy (Hansen's disease). It is an intracellular parasite, taking up residence in Schwann cells where, in due course, it triggers an autoimmune attack on them that leads to their destruction. The resulting loss of sensation makes it difficult to avoid injury to the extremities. *M. leprae* is a mycobacterium and a close relative of ***M. tuberculosis***, the cause of TB.

M. leprae infection also occurs naturally in the wild armadillos living in a band of southern states extending from Alabama through Texas. A survey of 39 human leprosy patients in that region revealed 25 of them infected with the identical strain found in the local armadillos. Although it was the first bacterium to be identified as a cause of human disease (in 1873), no bacteriologist has ever succeeded in cultivating it in vitro. It can, however, be propagated (slowly) in the nine-banded armadillo, and this has provided enough material to sequence its entire genome.

Its sequence, which was published in the 22 February 2001 issue of **Nature** (Cole, S. T. *et al.*) — when compared to that of ***M. tuberculosis*** — provides a vivid demonstration of degeneracy at the level of the genes. Although its genome is only about 25% smaller than that of *M. tuberculosis*, it has only 40% of the genes of its cousin. Many of the missing genes are still detectible, but they are now **pseudogenes** — genes that have mutated so that they can no longer be expressed in a protein product.

	M. tuberculosis	*M. leprae*
Size of genome (bp)	4,411,532	3,268,203
Protein-coding genes	3,959	1,604
citrate synthase genes (for citric acid cycle)	3	1
pyruvate dehydrogenase genes (for citric acid cycle)	6	2
lactate dehydrogenase genes (cellular respiration)	2	1
phosphofructokinase genes (glycolysis)	2	1

M. leprae is not an exception. The many bacterial genomes that have now been sequenced show that bacteria that are obligate intracellular parasites express far fewer proteins than bacteria that can live on culture medium.

A collection of links to examples of parasites

- **Viruses**
  - Retroviruses, incl HIV-1, the cause of AIDS
  - Influenza
  - an assortment of others
- **Bacteria**, the agents of
  - tetanus, botulism, and anthrax
  - typhoid, cholera, and plague
  - staphylococcal and streptococcal infections
  - gonorrhea
  - tuberculosis, leprosy, and diphtheria
  - syphilis and Lyme disease
  - typhus fever and Rocky Mountain spotted fever
- **Protists**
  - Plasmodium (agents of malaria)
  - Trypanosomes
- **Invertebrates**
  - Tapeworms
  - Blood flukes
- **Games Parasites Play** (some interesting interactions between host and parasite).

The Evolution of Symbiosis

It seems plausible that what begins as a parasitic relationship might over the course of time evolve into a mutualistic one as the two organisms evolve to minimize the damage to the host.

And there is some evidence for this. In 1966, K. W. Jeon discovered a culture of amoebas that had become infected with bacteria (60,000 to 150,000 per cell). The infection slowed their rate of growth and made them much more fragile. But five years later, the amoebas still were infected but now no ill effects could be seen. Most interesting for our question, the amoebas — or at least their nuclei — had become dependent on the bacteria.

- When the nucleus was removed from an infected amoeba and replaced with one from a uninfected strain, the combination worked fine.
- But when the nucleus from an uninfected cell was replaced with one from an infected cell, the combination usually failed to survive.

Evidently, after 5 years, the nuclei had become dependent on a bacterial function (an enzyme produced by the bacteria but no longer by the host). What started as parasitism had evolved into mutualism (the bacteria could not be grown outside their host). But it doesn't always work like that. There are other examples where a mutualistic relationship seems to have evolved into a commensalistic or even parasitic one. Some parasitic fungi seem to have evolved from ancestors living in the mutualistic

partnership of a lichen.

Some of the bacteria living in our large intestine supply us with vitamin K, thus evolving from commensalism to mutualism.

Mutually beneficial symbiotic relationships can lead to "degeneracy" also. Some marine annelid worms have completely lost the digestive tract of their relatives (like the common earthworm). One species gets its nourishment from a large population of at least 5 different species of bacteria living underneath its outer skin. The most abundant of these are **chemoautotrophs** (but these bacteria are gamma- and delta- proteobacteria not beta-proteobacteria) that manufacture food from carbon dioxide using the energy provided by oxidizing inorganic substances (H_2S, H_2) in the sediments in which the worm lives.

The nature of a symbiotic relationship can also change as circumstances change. Some fungi, bacteria, and protozoans that live harmlessly in most of us can cause **opportunistic infections** — that is become parasitic — in immunodeficient people, e.g., those with AIDS.

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17.4B: Insecticides

Inorganics

Inorganic compounds of arsenic, such as **lead arsenate**, have long been used against insect pests. However, these materials are highly toxic to nontarget organisms and persist in the environment. (Years after apple growers stopped using lead arsenate, high concentrations of lead can still be found in orchard soils.)

Botanicals

These are organic molecules (or mixtures) extracted from plants. Popular examples:

- **pyrethrins**
- **rotenone**
- **nicotine**
- **azadirachtin**. This extract from the neem tree affects insect growth and is discussed further under growth regulators.

Features:

- very toxic to insects
- relatively harmless to other organisms (except fish!)
- decompose readily so residues do not accumulate on crops or in the soil
- expensive, but you can get
- more bang for the buck by adding a **synergist** like **piperonyl butoxide**. Synergists have little toxicity themselves but enhance the effectiveness of the insecticide with which they are mixed.

Pyrethroids

Pyrethrins break down so rapidly in sunlight that they are of little use outdoors on crops. However, a number of synthetic pyrethrin-like substances — called **pyrethroids** — do not have this defect and are effective.

Example:

- permethrin (Ambush®, Pounce®)

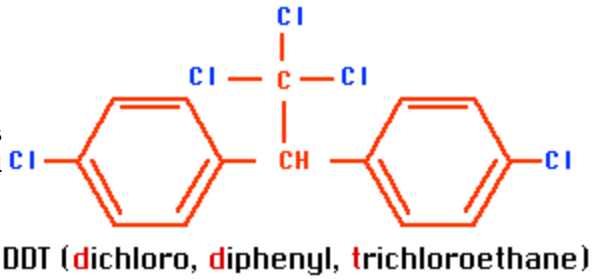
Bacillus thuringiensis (B.t.)

This bacterium parasitizes many caterpillars. Its spores and/or mixtures of its protein toxins are now being used against a variety of insect pests. Examples:

- Dipel®
- Javelin®
- Agree®

These are all stomach poisons; that is, they must be ingested to work.

Chlorinated Hydrocarbons

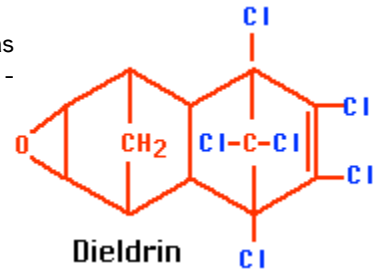


DDT (dichloro, diphenyl, trichloroethane)

DDT was the first of a long line of insecticides based on hydrocarbons with chlorine atoms replacing some of the hydrogen atoms. Its chemical name is dichloro, diphenyl, trichloroethane (see figure).

Some others:

- methoxychlor
- dieldrin (see figure)
- dicofol (Kelthane®)
- endosulfan (Thiodan®, Phaser®)



Dieldrin

DDT was introduced during World War II and, along with penicillin and the sulfa drugs, was responsible for the fact that this was the first war in history where trauma killed more people - combatants and noncombatants alike - than infectious disease.

DDT is effective against

- vectors of human diseases such as
  - malaria and yellow fever (both transmitted by mosquitoes)
  - plague (transmitted by fleas)
- many crop pests

Prior to the introduction of DDT, the number of cases of malaria in Ceylon (now Sri Lanka) was more than a million a year. By 1963 the disease had been practically eliminated from the island. However, growing concern about the hazards of DDT led to its abandonment there in the mid-1960s, and soon thereafter malaria became common once again.

DDT was especially effective against malarial mosquitoes because of its persistence and resistance to breakdown in the environment. One or two sprays a year on the walls of homes kept them free of mosquitoes. But DDT has several serious drawbacks.

Insecticide resistance

As early as 1946, Swedish workers discovered populations of houseflies resistant to DDT. This was quickly followed by many other reports of developing resistance. Other chlorinated hydrocarbons (like dieldrin and methoxychlor) were developed as substitutes, but in time insects developed resistance to these as well.

Persistence

DDT is stable and fat soluble. These properties cause it to accumulate in fat tissue. People who were heavily exposed to DDT (during its manufacture or application) often showed concentrations of DDT in their fat 1000 times higher than that in their blood. Even these high levels were probably of little harm to the workers. In the early stages of exposure, the blood levels of DDT (and its metabolite DDE) rise rapidly at first and then reach a steady level. From that point on, the body excretes it as fast as it acquires it.

Biomagnification

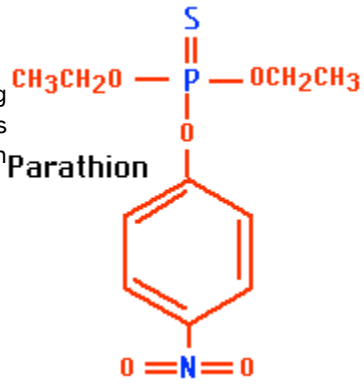
Although no harmful effects from average exposures to DDT have been seen in humans, DDT and other chlorinated hydrocarbons have been shown to harm other species, such as fishes, earthworms, and robins. The hazard of DDT to nontarget animals is particularly acute for those species living at the top of food chains. Carnivores at the ends of long food chains (e.g., ospreys, pelicans, falcons, and eagles) once suffered serious declines in fecundity and hence in population size because of this. High levels of chlorinated hydrocarbons interfere with forming eggshells of normal thickness.

Correlation between DDE concentrations in the eggs of Alaskan falcons and hawks and reduction in the thickness of their eggshells (compared with shells collected prior to 1947). DDE is a metabolite of DDT. Data from T. J. Cade, et. al., Science 172:955, 1971.

Species	Location	Average Concentration of DDE in Eggs (ppm)	Reduction in Shell Thickness
Peregrine falcon	Alaskan tundra (north slope)	889	-21.7%
Peregrine falcon	Central Alaska	673	-16.8%
Peregrine falcon	Aleutian Islands	167	-7.5%
Rough-legged hawk	Alaskan tundra (north slope)	22.5	-3.3%
Gyrfalcon	Seward Peninsular, Alaska	3.88	0

Another group of nontarget victims of DDT (and other pesticides) are insects that prey upon insect pests; that is, the **natural enemies** of the pests. Killing these has serious ecological and economic effects. For example, once apple growers began controlling pests with DDT, they quickly found their orchards being attacked by scale insects and mites. The reason: DDT had killed off their natural enemies.

Organophosphates



The organophosphates, e.g., **parathion** (right), are related to the nerve gases developed during World War II. They react irreversibly with the enzyme **acetylcholinesterase**, which is responsible for inactivating acetylcholine (ACh) at neuromuscular junctions and at certain synapses in the central and peripheral nervous systems.

Some other examples:

- malathion
- diazinon
- phosmet (Imidan®)
- chlorpyrifos (Lorsban®)

Some of the organophosphates are very toxic. Parathion, for example, is 30 times more toxic than DDT. Each year organophosphates poison thousands of humans throughout the world, causing hundreds of deaths. Medical personnel caring for poisoning victims are also at risk. They may be seriously poisoned by the excretions of, and even the vapors emanating from, their patients.

This table gives the LD_{50} values for some insecticides. In each case, the chemical was fed to laboratory rats. Note that the lower the LD_{50}, the more toxic the chemical.

Chemical	Category	Oral LD_{50} in Rats (mg/kg)
Aldicarb ("Temik")	Carbamate	1
Carbaryl ("Sevin")	Carbamate	307
DDT	Chlorinated hydrocarbon	87
Dieldrin	Chlorinated hydrocarbon	40
Diflubenzuron ("Dimilin")	Chitin inhibitor	10,000
Malathion	Organophosphate	885
Methoprene	JH mimic	34,600
Methoxychlor	Chlorinated hydrocarbon	5,000
Parathion	Organophosphate	3

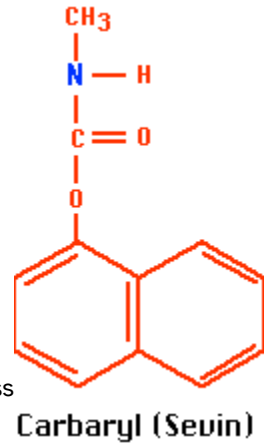
Chemical	Category	Oral LD_{50} in Rats (mg/kg)
Piperonyl butoxide	Synergist	7,500
Pyrethrins	Plant extract	200
Rotenone	Plant extract	60

Unlike chlorinated hydrocarbons,

- Organophosphates break down quickly in the environment, and thus residues on crops are less likely to be a problem.
- They are not stored in animal tissue, so biomagnification has not been a problem either.

For these reasons, their use has greatly reduced the hazard to nontarget species like ospreys and eagles (at the price of a much greater hazard to humans). Development of resistance is just as much a problem as it is with the chlorinated hydrocarbons. The carbamates were introduced in an attempt to keep ahead.

Carbamates



Carbaryl (Sevin)

Carbamate insecticides are also inhibitors of acetylcholinesterase, but their action is reversible.

Some examples:

- **carbaryl** (Sevin®)
- aldicarb (Temik®)
- methomyl (Lannate®)

Features:

- These compounds are rapidly detoxified and excreted so their risk to warm-blooded animals is less than the other agents we have looked at.
- They are degraded rapidly in the environment so persistence is not a problem.
- They are, however, a danger to many useful insects, especially honeybees.

Growth Regulators

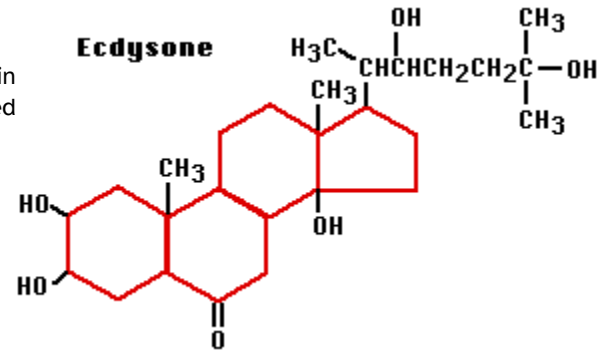
The members of this diverse group interfere in one way or another with insect development. Although most insect growth regulators do not affect adults, for many pests, it is the larval stages that are the most destructive.

Chitin Inhibitors

These substances, diflubenzuron (Dimilin®) is an example, interfere with the synthesis of **chitin**, the material that makes up the insect exoskeleton. It seems to have very low toxicity for vertebrates, but is harmful to crustaceans as well as insects. Its effect on fungi, which also synthesize chitin, needs to be studied.

Molting Disruptors

Insects must periodically shed their exoskeleton — called molting — in order to grow. Each molt is triggered by a steroid hormone called **ecdysone**.



A few synthetic ecdysone

- agonists, e.g.,
  - tebufenozide (Confirm®)
  - methoxyfenozide (Intrepid®)
- inhibitors, e.g., azadirachtin (Neemix®)

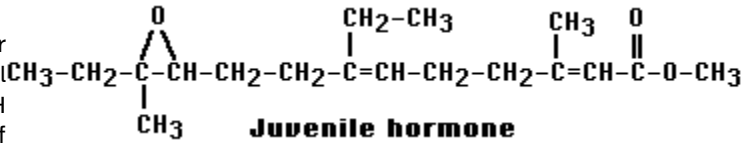
are now used as insecticides.

Juvenile Hormone (JH)

In some insects, the final molt produces an adult that looks pretty much like the earlier larval stages. In others, like moths and butterflies, the final molt of the larva (caterpillar) produces a **pupa**. After a period of **metamorphosis**, the adult moth emerges.

Ecdysone triggers larva-to-larva molts as long as another hormone, called **juvenile hormone** (**JH**), is present. In its absence, ecdysone promotes the pupa-to-adult molt. Thus normal metamorphosis seems to occur when the output of JH diminishes spontaneously in the mature caterpillar.

When solutions of JH are sprayed on mature caterpillars, or on the foliage upon which they are feeding, their normal development is upset. This raises the possibility of using JH as an insecticide (one that might avoid the problem of developing resistance).



As it turns out, JH is too unstable to be practical, but some synthetic **JH mimics**, e.g.,

- methoprene (Altosid®)
- pyriproxyfen (Esteem®, Knack®, Distance®)
- diofenolan

are now being used.

Precocenes

These are substances, first discovered in plants, that damage the **corpora allata** thus preventing juvenile hormone (JH) from doing its normal job. Applied to early larval stages, precocenes induce premature or precocious metamorphosis (like that induced by the surgical removal of the corpora allata). Not only does precocious metamorphosis cut short the destructive larval phase of the insect, but the adults are abnormal (besides being small). The females, for example, are sterile (because JH is needed for development of the ovaries).

Summary

It is often said that the evolution of insecticide resistance requires the introduction of ever more powerful insecticides. But what is really needed is a new substance that attacks some other chink in the insect's armor. Whether gram for gram the new substance is more or less toxic to the insect (and whether its LD_{50} for mammals is lower or higher) is quite a separate issue.

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17.4C: Biological Control of Pests

The biological control of pests involves using natural enemies of the pest to control it — instead of chemical agents like insecticides and herbicides. Not only should this be safer for the environment, but once established - the natural enemies might be able to sustain their population avoiding the need for future treatments.

Most of the species that we consider pests are plants ("weeds") or animals (especially insects) that have invaded a new habitat without being accompanied by the natural enemies that kept them in check in their original home. With increasing international travel and trade, this problem becomes increasingly severe.

The Biological Control of Insects

Cottony Cushion Scale Insect

In 1887, this insect - an import from Australia, was devastating the citrus groves of California. A U.S. entomologist went to Australia to find a natural enemy and came back with the **vedalia beetle**, a species of lady beetle. Released in California, the beetle quickly brought the scale under control.

At least until 1946. In that year the pest made a dramatic comeback. This coincided with the first use of DDT in the groves. DDT not only killed the target pest insects but the vedalia beetle as well. Only by altering spray procedures and reintroducing the beetle was the scale insect again controlled.

The Sterile Male Technique

This technique was first applied against the **screwworm fly**, a serious pest of cattle. The female flies lay their eggs in sores or other open wounds on the animals. After hatching, the larvae eat the tissues of their host. As they do so, they expose a still larger area to egg laying, often finally killing the host.

Prior to its eradication from the southeastern United States, the screwworm was causing huge annual livestock losses. The sterile male technique involves releasing factory-reared and sterilized flies into the natural population. Sterilization is done by exposing the factory flies to just enough gamma radiation to make them sterile but not enough to reduce their general vigor.

Starting in early 1958, up to 50 million sterilized flies were released each week from aircraft flying over Florida and parts of the adjoining states. Each time a fertile female in the natural population mated with a sterile male, the female layed sterile eggs. Since the females mate only once, her reproductive career was at an end. By early 1959, the pest was totally eliminated east of the Mississippi River. Success depended only on the sterile males. In fact, the presence of sterile females was a drawback (because they competed with the intended target), but it was difficult to separate the sexes.

The southwestern states presented a harder problem because the fly winters in Mexico and with each new season could move across the border. Even so, by expanding the program to include Mexico as well, the screwworm fly was finally eliminated from both countries by 1991.

The sterile male technique has also been used with success against several other insect pests, including

- The "medfly", a destructive fruit fly (not Drosophila) in California
- The tsetse fly, the vector of African sleeping sickness.

Using Genetic Engineering to Improve the Sterile Male Technique

There are two problems with the sterile male technique

- The factory produces both males and females in equal numbers. But if you release the females along with the males, many males will mate with them rather than with wild females. For this reason, the sexes are now separated - an expensive operation - and only males released.

- Irradiation may harm the males in subtle ways - reducing their breeding effectiveness.

Genetic engineering may solve both these problems.

A group of British entomologists (see Thomas, D. D., et al., in the 31 March 2000 issue of **Science**) have engineered **Drosophila** so that

- Only males are produced.
- When these mate with normal females, the females give birth only to males (thus ending the population).

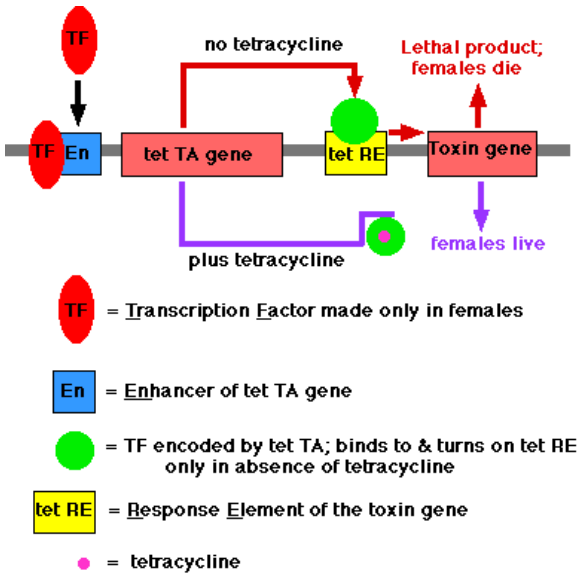


Figure 17.4.3.1 Genetic engineering to improve male sterile techniques

The system works like this:

- Transgenic flies are created containing a chromosome with an enhancer (**En**) for a gene (***tet TA***) that encodes a transcription factor (green disk) that binds the response element (**tet RE**), which is part of the promoter for a gene (**Toxin gene**) encoding a protein whose product is lethal to the insect.
- **Only females** produce the transcription factor that binds **En**.
- If the antibiotic tetracycline is given to the insects, it binds to the tet TA transcription factor, producing an allosteric change that prevents the transcription factor from binding the tetracycline response element (tet RE).
- The toxin gene is repressed and viable females are made.
- In the **absence** of tetracycline, the tet TA transcription factor (green disk) **turns on** the toxin promoter and no females are produced.
- Because the tet TA enhancer (En) responds to a transcription factor made only by females, males are produced whether tetracycline is present or not.

If this system could be applied to an insect pest (and most seem to produce the same female-specific transcription factor [red oval]),

- removing tetracycline from the food of a batch of flies in the factory would produce a new generation containing only males.
- Released into the wild, these would pass their transgenic chromosome on to the offspring of all the wild females they mated with.
- The genes are dominant so even though the next generation would be heterozygous, only males would be produced.
- Thus the pest population would soon die out.

In 2010, release of male mosquitoes *Aedes aegypti*, the vector of dengue fever - genetically modified (GM) with a similar system reduced the resident mosquito population in part of Grand Cayman (Caribbean) by 80%.

Gene Drive

Techniques have now been developed which greatly increase the frequency of any desired gene in a population. So far, the uncertainties of quickly spreading an engineered gene through an entire wild population has kept the process strictly confined to the laboratory. The process, called gene drive or the **mutagenic chain reaction**, is described on a separate page.

Male Confusion

Insect sex attractants have also been enlisted in the fight against harmful insects. Distributing a sex attractant throughout an area masks the female's own attractant so the sexes fail to get together. This is called "communication disruption" or "male confusion". In some cotton-growing areas, male confusion with the sex attractant of the pink bollworm reduced the need for conventional chemical insecticides by 90%. It has been used successfully against pests that attack tomatoes, grapes, and peaches.

Parasites vs Insect Pests

Parasites, as well as predators, have been used to achieve control over destructive insects.

- The bacterium ***Bacillus popilliae*** is supplied commercially to help control the Japanese beetle by infecting it with "milky disease".
- ***Bacillus thuringiensis*** ("**Bt**") is sold commercially to aid in controlling a number of harmful insects. In some cases, the bacterium itself infects the pests and eventually kills them. But in most cases, it is the toxin manufactured by the bacterium while it is growing in culture that does the job.

The Biological Control of Plants

Prickly-pear Cactus (Opuntia)

Introduced into Australia, this cactus soon spread over millions of hectares of range land driving out forage plants. In 1924, the cactus moth, ***Cactoblastis cactorum***, was introduced (from Argentina) into Australia. The caterpillars of the moth are voracious feeders on prickly-pear cactus, and within a few years, the caterpillars had reclaimed the range land without harming a single native species. However, its introduction into the Caribbean in 1957 did not produce such happy results. By 1989, the cactus moth had reached Florida, and now threatens 5 species of native cacti there.

Klamath Weed

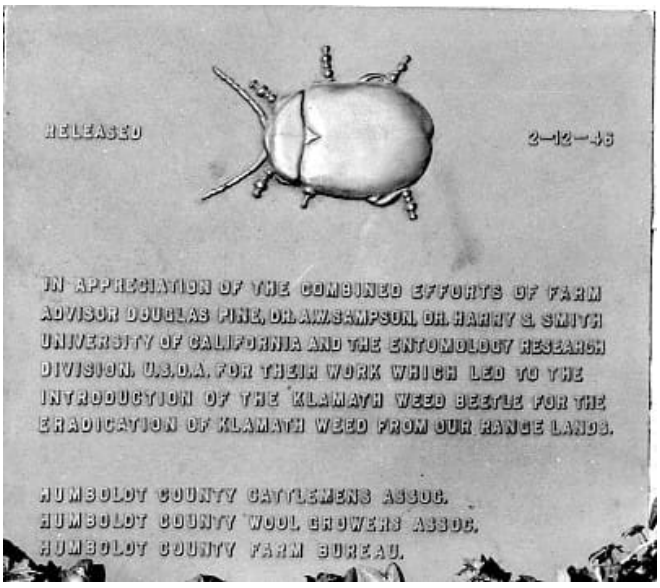


Figure 17.4.3.2

In 1946 two species of Chrysolina beetles were introduced into California to control the Klamath weed (also known as St. Johnswort, and the same plant that yields the popular herbal concoction) that was ruining millions of acres of range land in California and the Pacific Northwest. Before their release, the beetles were carefully tested to make certain that they would not turn to valuable plants once they had eaten all the Klamath weed they could find.

The beetles succeeded beautifully, restoring about 99% of the endangered range land and earning them a commemorative plaque at the Agricultural Center Building in Eureka, California. (Photo courtesy of John V. Lenz.)

Rules to Live By

- Pick only candidates that have a very narrow target preference; i.e., eat only a sharply-limited range of hosts
- Test each candidate carefully to be sure that once it has cleaned up the intended target, it doesn't turn to desirable species.
- Don't use bio controls against native species.
- Avoid introducing alien species into the environment.

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UNIT 18: EVOLUTION

18.1: Evolution and Adaptation

18.2: Speciation

18.3: The Evolution of Body Form in Animals

18.4: Recapitulation

18.5: Mutation and Evolution

18.6: The Hardy-Weinberg Equilibrium

18.7: Polymorphisms

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18.9: The Origin of Life

18.10: Mars

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18.12: Geologic Eras

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18.1: Evolution and Adaptation

In 1859 the English naturalist Charles Darwin published The Origin of Species. The book contained two major arguments: First, Darwin presented a wealth of evidence of evolution. He said that all living things on earth today are the descendants - with modifications - of earlier species. Second, he proposed a mechanism - natural selection - to explain how evolution takes place.

Evolution involves two interrelated phenomena: adaptation and speciation. In adaptation, over the course of time, species modify their phenotypes in ways that permit them to succeed in their environment. In speciation, over the course of time, the number of species multiplies; that is, a single species can give rise to two or more descendant species. In fact, Darwin maintained that all species are related; that is, any two species on earth today have shared a common ancestor at some point in their history.

Natural Selection

- Living things produce more offspring than the finite resources available to them can support.
- Thus living things face a constant struggle for existence.
- The individuals in a population vary in their phenotypes.
- Some of this variation is inheritable; that is, it is a reflection of variations in genotype.
- Those variants best adapted to the conditions of their life are most likely to survive and reproduce themselves ("survival of the fittest").
- To the extent that their adaptations are inheritable, they will be passed on to their offspring.

The forces of natural selection act on phenotypes, but only if there is a change in the genotypes of a population has evolution occurred.

The Measure of "Fitness"

Fitness is a measure of reproductive success. Those individuals who leave the largest number of mature offspring are the fittest. This can be achieved in several ways:

- Survival (mortality selection)
- Mating success (sexual selection)
- Family size (fecundity selection)

Survival

Any trait that promotes survival - at least until one's reproductive years are over - increases fitness. Such traits are adaptations.

Sexual Selection

In sexual selection, one sex - usually the female - chooses among the available males. Any inherited trait that improves the mating success of certain individuals will become more pronounced in succeeding generations. Some examples:

- When ready to mate, female three-spined sticklebacks (fish) choose males with many Class II MHC alleles over males with fewer alleles. Class II alleles encode the proteins that present antigens to the immune system. Presumably, the more of them you have, the greater the diversity of parasite antigens your immune system can recognize and defend against.The females distinguish between the males by soluble molecules ("odors") the males release into the water. How these "odors" are controlled by the MHC alleles is not known.
- A culture of Drosophila set up with equal numbers of red-eyed and white-eyed flies of both sexes will, after 25 generations or so, end up having only red-eyed (the "normal") flies in it. This despite the fact that white-eyed flies are just as healthy and live just as long as red-eyed flies, i.e., they are equal in terms of survival. But, as it turns out, not only do red-eyed females prefer red-eyed males, but white-eyed females do also.

In other cases of sexual selection, one phenotype prefers to mate with others of the same phenotype. This is called assortative

mating.

Fecundity Selection

The production of a large number of mature offspring is a measure of fitness. I stress mature because only they can pass these traits on to another generation. Some ways to do this:

- Earlier breeding. If some females become sexually mature earlier than others, their chances of leaving offspring are enhanced.
- For some species (e.g., fish, oysters), which provide little or no care for their young, fitness is measured by the number of fertilized eggs they produce.
- For species (such as ourselves) that take care of their young, selection acts to reduce family size (to a point). A large study in Utah (U.S.A) showed that in the 19th century, families with fewer children had more surviving grandchildren.

All the forces of natural selection outlined above work on individuals. But there is an increasing body of evidence that natural selection can also act on groups. Natural selection that appears to work counter to the benefit of some individuals while enhancing the prospects of their relatives is called kin selection. It is discussed on a separate page.

Are Humans Exempt from Natural Selection?

It has been argued that advances in medicine, sanitation, etc. have removed humans from the rigors of natural selection. There is probably some truth to this, but consider that of all the human eggs that are fertilized, fewer than half will ever reproduce themselves. The others are eliminated as follows:

- Mortality selection
  - Approximately 30% of pregnancies end by spontaneous abortion of embryos and fetuses or by stillbirth.
  - Death in infancy and childhood claims another 5% or more.
- Sexual selection
  - Another 20% will survive to adulthood but never marry.
- Fecundity selection
  - Of those that do marry, 10% will have no children.

Continuous Variation

Most traits in a population such as height and body weight vary in a continuous way from one extreme to the other.

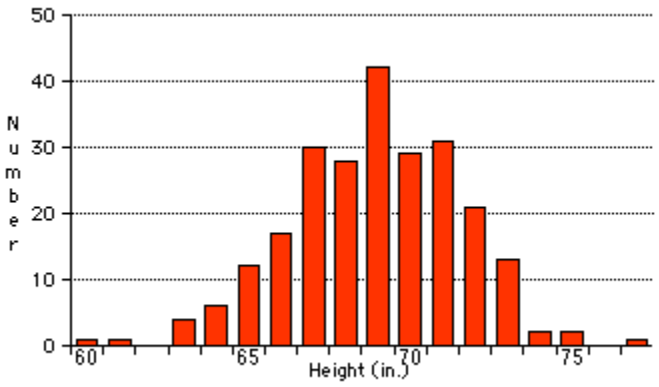


Figure 18.1.1 Distribution of trait in population

A plot of the distribution of the trait in a population often produces a bell-shaped curve like this one that shows the distribution of heights among a group of male secondary-school seniors. Such a distribution could arise from environmental factors - perhaps the continuous height variation in the boys is simply a result of variation in their diet as they grew up or genetic factors - tall parents tend to have tall children or - most likely - both.

Heritability

One can sort out the relative contribution of genetic and environmental factors by comparing the range of a trait in the offspring compared with the average value of that trait in their parents. If the offspring of selected parents occupy the same range as the entire population, environmental factors are working alone. The trait has a zero heritability. Example: The length of the seeds of a pure strain of beans may vary over several millimeters. However, if extra-large beans are mated, the new crop shows no shift to a larger size. So the heritability of length is zero. On the other hand, if the offspring of two extra-large mice are just as large as they are, genes are probably at work. The trait is said to have a heritability of 1.

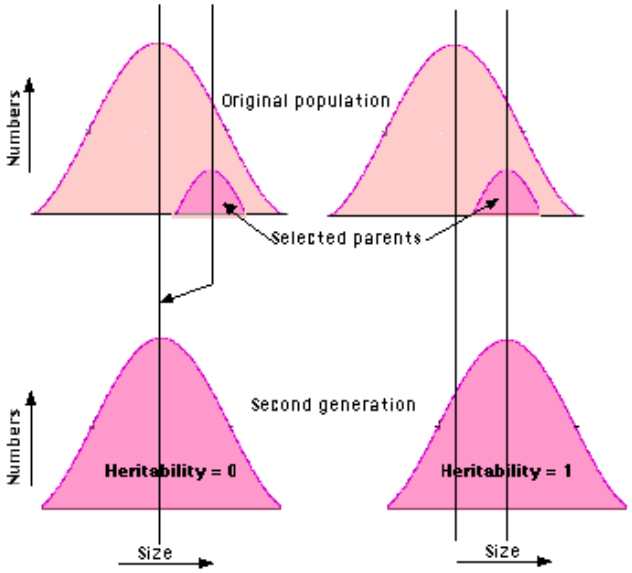


Figure 18.1.2 Heritability

The Effects of Selection on Populations

The pressures of natural selection can affect the distribution of phenotypes in a population in several ways.

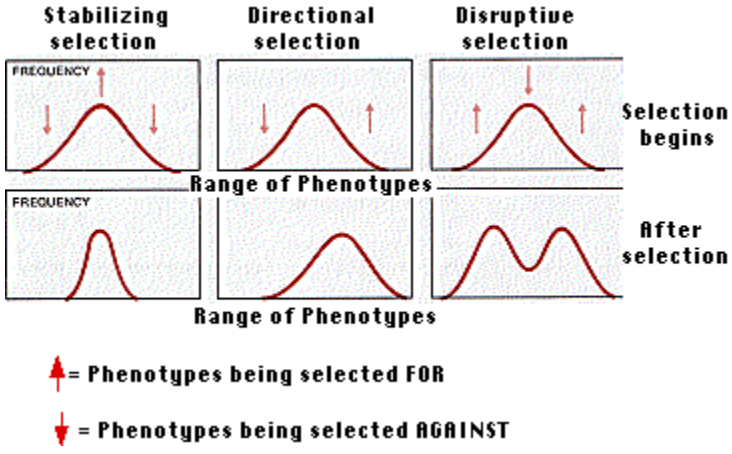


Figure 18.1.3 Effects of selection on population

Stabilizing Selection

Natural selection often works to weed out individuals at both extremes of a range of phenotypes resulting in the reproductive success of those near the mean. In such cases, the result is to maintain the status quo. It is not always easy to see why both extremes should be handicapped; perhaps sexual selection or liability to predation is at work. In any case, stabilizing selection is common. In humans, for example, the incidence of infant mortality is higher for very heavy as well as for very light babies.

Directional Selection

A population may find itself in circumstances where individuals occupying one extreme in the range of phenotypes are favored over the others. Since 1973, Peter and Rosemary Grant - aided by a succession of colleagues - have studied Darwin's finches in the Galapagos Islands. When rainfall, and thus food, are plentiful, the ground finches tend to have a varied diet, e.g., eat seeds of a range of sizes and show considerable variation in body and beak size (large beaks are better for large seeds but can handle small seeds as well as small beaks).

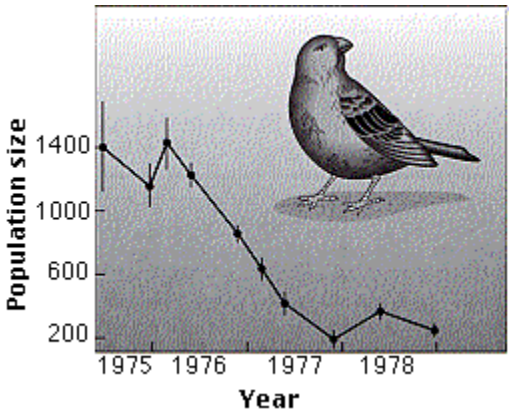


Figure 18.1.4 Geo fortis

From 1976 through 1977, a severe drought struck the islands, with virtually no rainfall for over a year. This caused a precipitous decline in the production of the seeds that are the dietary mainstay of *Geospiza fortis*, the medium ground finch. The graph (from P. T. Boag and P. R. Grant in Science 214:82, 1981) shows how the population declined from 1400 to 200 on the island of Daphne Major, a tiny (10-acre = 4 hectares) member of the Galapagos Islands.

One of the plants to make it through the drought produces seeds in large, tough fruits that are virtually impossible for birds with a beak smaller than 10.5 mm to eat. Sampling the birds that died as well as those that survived showed that he larger birds were favored over the smaller ones and Those with larger beaks were favored over those with smaller ones.

	Beak length (mm)	Beak depth (mm)
Dead birds	10.68	9.42
Survivors	11.07	9.96

Here, then was natural selection at work. But did it produce evolution? The answer turned out to be yes. As the population of G. fortis recovered after the rains returned, the average body size and beak depth of their offspring was greater than before (an increase of 4–5% for beak depth). The bell-shaped curve had been shifted to the right — directional selection.

More recently, the Grants and colleagues at Harvard Medical School have shown that

- beak width and depth in the ground finches are correlated with the timing and intensity of expression of the gene, *Bmp4*, (that encodes bone morphogenetic protein-4) in the tissue that will form the upper beak. *Bmp4* expression appears earlier in development and with greater intensity in the large-beaked *Geospiza magnirostris* (the large ground finch) than in its smaller-beaked relatives, *Geospiza fortis* (the medium ground finch) and *Geospiza fuliginosa* (the small ground finch). See Abzhanov, A., *et al*., Science, 3 September 2004.
- However, beak length is correlated with the intensity of expression of the gene *CaM* that encodes the Ca^{2+}-binding protein calmodulin in the tissue that will form the upper beak. *CaM* expression is much higher in the embryonic tissue of the cactus finches (*G. scandens* and *G. conirostris* - both with long beaks) than in their short-beaked relatives, the ground finches *G. fortis* and *G. magnirostris*. (See Abzhanov, A., *et al*., Nature, 3 August 2006.)

Industrial Melanism

Many species of moths in the British Isles began to become darker in color in the 19th century. The best-studied example is the peppered moth, *Biston betularia*. The moth gets its name from the scattered dark markings on its wings and body. In 1849, a

coal-black mutant was found near Manchester, England. Within a century, this black form had increased to 90% of the population in this region. The moth flies at night and rests by day on tree trunks. In areas far from industrial activity, the trunks of trees are encrusted with lichens. As the photos show, the light form (circled in red) is practically invisible against this background.

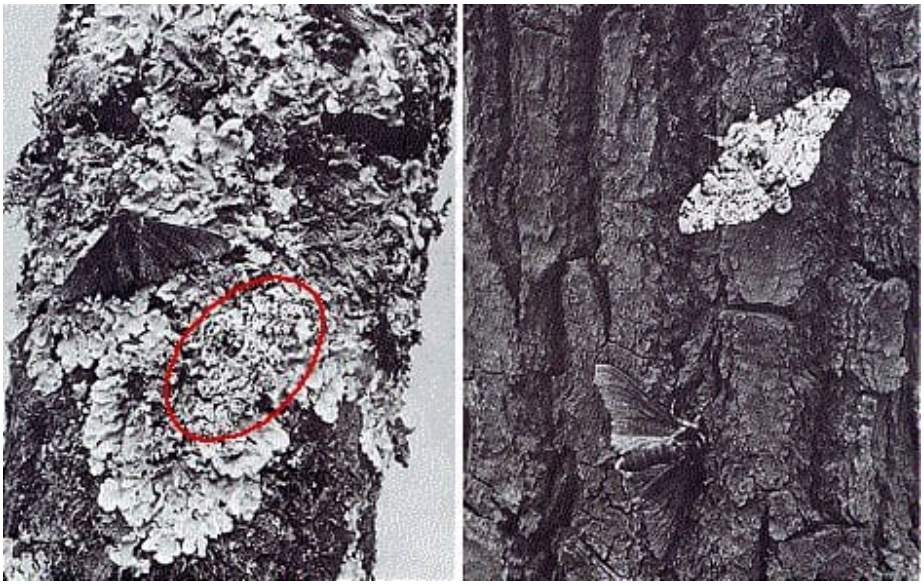


Figure 18.1.5 Peppered Moth

In areas where air pollution is severe, the combination of toxic gases and soot has killed the lichens and blackened the trunks. Against such a background, the light form stands out sharply. The moth is preyed upon by birds that pluck it from its resting place by day. In polluted woods, the dark form has a much better chance of surviving undetected. When the English geneticist H. B. D. Kettlewell (who supplied the photos) released moths of both types in the woods, he observed that birds did, indeed, eat a much higher fraction of the light moths he released than of the dark. Since pollution abatement programs were put in place after World War II, the light form has been making a comeback in the Liverpool and Manchester areas.

Disruptive Selection

In some circumstances, individuals at both extremes of a range of phenotypes are favored over those in the middle. This is called disruptive selection.

An example:

The residues ("tailings") of mines often contain such high concentrations of toxic metals (e.g., copper, lead) that most plants are unable to grow on them. However, some hardy species (e.g. certain grasses) are able to spread from the surrounding uncontaminated soil onto such waste heaps. These plants develop resistance to the toxic metals while their ability to grow on uncontaminated soil decreases. Because grasses are wind pollinated, breeding between the resistant and nonresistant populations goes on. But evidently, disruptive selection is at work. Higher death rates of both less resistant plants growing on contaminated soil and more resistant plants growing on uncontaminated soil leads to increasing divergence of the populations into two subpopulations with the extreme manifestations of this trait.

The evolutionary significance of disruptive selection lies in the possibility that the gene pool may become split into two distinct gene pools. This may be a way in which new species are formed. The formation of one or more species from a single precursor species is called speciation.

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18.2: Speciation

One of the best definition os species is that of the evolutionary biologist Ernst Mayr: "A species is an actually or potentially interbreeding population that does not interbreed with other such populations when there is opportunity to do so." However, sometimes breeding may take place (as it can between a horse and a donkey) but if so, the offspring are not so fertile and/or well adapted as the parents (the mule produced is sterile).

Allopatric Speciation: the Role of Isolation in Speciation

The formation of two or more species often (some workers think always!) requires geographical isolation of subpopulations of the species. Only then can natural selection or perhaps genetic drift produce distinctive gene pools. It is no accident that the various **races** (or "**subspecies**") of animals almost never occupy the same territory. Their distribution is **allopatric** ("other country").

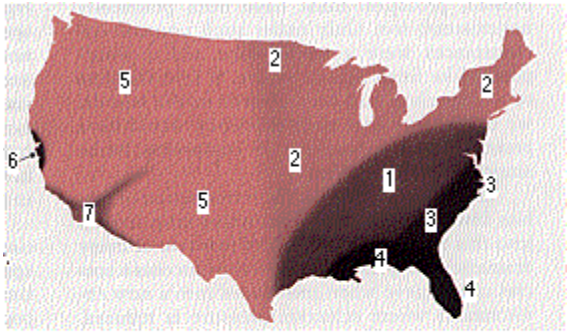


Figure 18.2.1 Yellowthroat

The seven distinct subspecies or races of the yellowthroat ***Geothlypis trichas*** (a warbler) in the continental U.S. would soon merge into a single homogeneous population if they occupied the same territory and bred with one another.

Darwin's Finches

As a young man of 26, Charles Darwin visited the Galapagos Islands off the coast of Ecuador. Among the animals he studied were what appeared to be 13 species* of finches found nowhere else on earth.

- Some have stout beaks for eating seeds of one size or another (#2, #3, #6).
- Others have beaks adapted for eating insects or nectar.
- One (#7) has a beak like a woodpecker's. It uses it to drill holes in wood, but lacking the long tongue of a true woodpecker, it uses a cactus spine held in its beak to dig the insect out.
- One (#12) looks more like a warbler than a finch, but its eggs, nest, and courtship behavior is like that of the other finches.

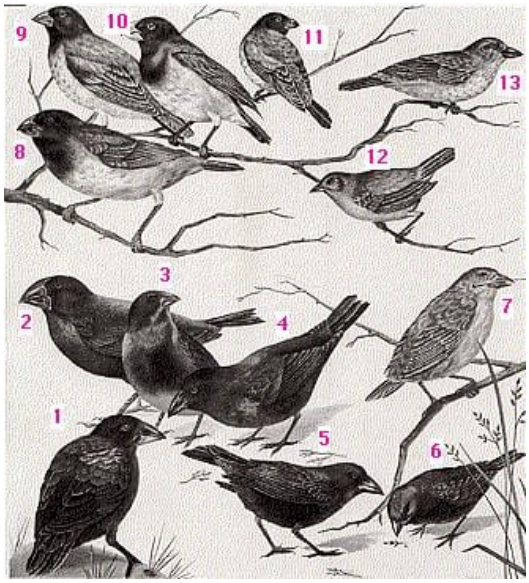


Figure 18.2.2 Darwin's finches

Darwin's finches. The finches numbered 1–7 are ground finches. They seek their food on the ground or in low shrubs. Those numbered 8–13 are tree finches. They live primarily on insects.

1. Large cactus finch (*Geospiza conirostris*)
2. Large ground finch (*Geospiza magnirostris*)
3. Medium ground finch (***Geospiza fortis***)
4. Cactus finch (*Geospiza scandens*)
5. Sharp-beaked ground finch (*Geospiza difficilis*)
6. Small ground finch (*Geospiza fuliginosa*)
7. Woodpecker finch (*Cactospiza pallida*)
8. Vegetarian tree finch (*Platyspiza crassirostris*)
9. Medium tree finch (***Camarhynchus pauper***)
10. Large tree finch (***Camarhynchus psittacula***)
11. Small tree finch (*Camarhynchus parvulus*)
12. Warbler finch (*Certhidia olivacea*)
13. Mangrove finch (*Cactospiza heliobates*)

(From BSCS, Biological Science: Molecules to Man, Houghton Mifflin Co., 1963)

* Genetic analysis provides evidence that:

- There are actually two species of warbler finch — *Certhidia olivacea* now called the green warbler finch and *Certhidia fusca*, the gray warbler finch.
- The various populations of *Geospiza difficilis* found on the different islands belong to one or another of three clades so genetically distinct that they deserve full species status.

Whether the number is 13 or 17, since Darwin's time, these birds have provided a case study of how a single species reaching the Galapagos from Central or South America could - over a few million years - give rise to the various species that live there today. Several factors have been identified that may contribute to speciation.

Ecological opportunity

When the ancestor of Darwin's finches reached the Galapagos, it found **no predators** (There were no mammals and few reptiles on the islands.) and **few, if any, competitors**. There were only a handful of other types of songbirds. In fact, if true warblers or woodpeckers had been present, their efficiency at exploiting their niches would surely have prevented the evolution of warblerlike and woodpeckerlike finches.

Geographical Isolation (allopatry)

The proximity of the various islands has permitted enough migration of Darwin's finches between them to enable distinct island populations to arise. But the distances between the islands is great enough to limit interbreeding between populations on different islands. This has made possible the formation of distinctive **subspecies** (**= races**) on the various islands.

The importance of geographical isolation is illuminated by a single, fourteenth, species of Darwin's finches that lives on **Cocos Island**, some 500 miles (800 km) to the northeast of the Galapagos. The first immigrants there must also have found relaxed selection pressures with few predators or competitors. How different the outcome, though. Where one immigrant species gave rise to at least 13 on the scattered Galapagos Islands, no such divergence has occurred on the single, isolated Cocos Island.

Evolutionary Change

In isolation, changes in the gene pool can occur through some combination of natural selection, genetic drift, and founder effect. These factors may produce distinct subpopulations on the different islands. So long as they remain separate (**allopatric**) we consider them **races** or **subspecies**. In fact, they might not be able to interbreed with other races but so long as we don't know, we assume that they could.

How much genetic change is needed to create a new species? Perhaps not as much as you might think. For example, changes at one or just a few gene loci might do the trick. For example, a single mutation altering flower color or petal shape could immediately prevent cross-pollination between the new and the parental types (a form of prezygotic isolating mechanism).

Reunion

The question of their status - subspecies or true species - is resolved if they ever do come to occupy the same territory again (become **sympatric**). If successful interbreeding occurs, the differences will gradually disappear, and a single population will be formed again. Speciation will not have occurred. If, on the other hand, two subspecies reunite but fail to resume breeding, speciation has occurred and they have become separate species.

An example: The medium tree finch ***Camarhynchus pauper*** is found only on **Floreana Island**. Its close relative, the large tree finch, ***Camarhynchus psittacula***, is found on all the central islands including Floreana. Were it not for its presence on Floreana, both forms would be considered subspecies of the same species. Because they do coexist and maintain their separate identity on Floreana, we know that speciation has occurred.

Isolating Mechanisms

What might keep two subpopulations from interbreeding when reunited geographically? There are several mechanisms.

Prezygotic Isolating Mechanisms act before fertilization occurs. Sexual selection - a failure to elicit mating behavior. On Floreana, *Camarhynchus psittacula* has a longer beak than *Camarhynchus pauper*, and the research teams led by Peter and Rosemary Grant have demonstrated that beak size is an important criterion by which Darwin's finches choose their mates. Two subpopulations may occupy different habitats in the same area and thus fail to meet at breeding time. In plants, a shift in the time of flowering can prevent pollination between the two subpopulations. Structural differences in the sex organs may become an isolating mechanism. The sperm may fail to reach or fuse with the egg.

Postzygotic Isolating Mechanisms act even if fertilization does occur. Even if a zygote is formed, genetic differences may have become so great that the resulting hybrids are less viable or less fertile than the parental types. The sterile mule produced by mating a horse with a donkey is an example. Sterility in the males produced by hybridization is more common than in females. In fact, it is the most common postzygotic isolating mechanism. When ***Drosophila melanogaster*** attempts to mate with its relative ***Drosophila simulans***, no viable males are even produced. Mutations in a single gene (encoding a component of the nuclear pore complex) are responsible.

Reinforcement

When two species that have separated in allopatry become reunited, their prezygotic and postzygotic isolating mechanisms

may become more stringent than those between the same species existing apart from each other. This phenomenon is called **reinforcement**. It arises from natural selection working to favor individuals that avoid interspecific matings, which would produce less-fit hybrids, when the two species are first reunited.

Speciation by Hybridization

Hybridization between related angiosperms is sometimes followed by a doubling of the chromosome number. The resulting **polyploids** are now fully fertile with each other although unable to breed with either parental type - a new species has been created. This appears to have been a frequent mechanism of speciation in angiosperms. Even without forming a polyploid, interspecific hybridization can occasionally lead to a new species of angiosperm. Two species of sunflower, the "common sunflower", *Helianthus annuus*, and the "prairie sunflower", *H. petiolaris*, grow widely over the western half of the United States. They can interbreed, but only rarely are fertile offspring produced.

However, Rieseberg and colleagues have found evidence that successful hybridization between them has happened naturally in the past. They have shown that three other species of sunflower (each growing in a habitat too harsh for either parental type) are each the product of an ancient hybridization between *Helianthus annuus* and *H. petiolaris*. Although each of these species has the same **diploid** number of chromosomes as the parents (2**n** = 34), they each have a pattern of chromosome segments that have been derived, by **genetic recombination**, from both the parental genomes. They demonstrated this in several ways, notably by combining RFLP analysis with the polymerase chain reaction (PCR).

They even went on to produce (at a low frequency) *annuus* x *petiolaris* hybrids in the greenhouse that mimicked the phenotypes and genotypes of the natural hybrids. (These monumental studies were described in the 29 August 2003 issue of **Science**.)

Another example. In Pennsylvania, hybrids between a species of fruit fly (not Drosophila) that feeds on blueberries and another species (again, not Drosophila) that feeds on snowberries feed on honeysuckle where they neither encounter competition from their parental species nor have an opportunity to breed with them (no introgression). This study was published in the 28 July 2005 issue of **Nature**. So speciation can occur as a result of hybridization between two related species, if the hybrid

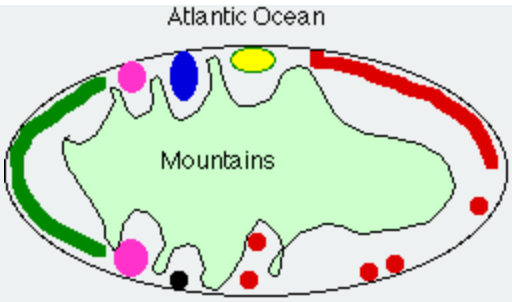
- receives a genome that enables it to breed with other such hybrids but not breed with either parental species,
- can escape to a habitat where it does not have to compete with either parent,
- is adapted to live under those new conditions.

Adaptive Radiation

The processes described in this page can occur over and over. In the case of Darwin's finches, they must have been repeated a number of times forming new species that gradually divided the available habitats between them. From the first arrival have come a variety of ground-feeding and tree-feeding finches as well as the warblerlike finch and the tool-using woodpeckerlike finch. The formation of a number of diverse species from a single ancestral one is called an **adaptive radiation**.

Speciateion in theHouse mice on the island of Madeira

A report in the 13 January 2000 issue of **Nature** describes a study of house mouse populations on the island of Madeira off the Northwest coast of Africa. These workers (Janice Britton-Davidian et al) examined the karyotypes of 143 house mice (***Mus musculus domesticus***) from various locations along the coast of this mountainous island.



Schematic representation of the Portuguese island of Madeira showing (in separate colors) the distribution of 6 races of house mice.

Figure 18.2.3 Madeira mice

Their findings:

- There are 6 distinct populations (shown by different colors)
- Each of these has a distinct karyotype, with a diploid number less than the "normal" (2**n** = 40).
- The reduction in chromosome number has occurred through **Robertsonian fusions**. Mouse chromosomes tend to be **acrocentric**; that is, the centromere connects one long and one very short arm. Acrocentric chromosomes are at risk of translocations that fuse the long arms of two different chromosomes with the loss of the short arms.
- The different populations are **allopatric**; isolated in different valleys leading down to the sea.
- The distinct and uniform karyotype found in each population probably arose from genetic drift rather than natural selection.
- The 6 different populations are technically described as **races** because there is no opportunity for them to attempt interbreeding.
- However, they surely meet the definition of true species. While hybrids would form easily (no prezygotic isolating mechanisms), these would probably be infertile as proper synapsis and segregation of such different chromosomes would be difficult when the hybrids attempted to form gametes by meiosis.

Sympatric Speciation

Sympatric speciation refers to the formation of two or more descendant species from a single ancestral species all occupying the same geographic location. Some evolutionary biologists don't believe that it ever occurs. They feel that interbreeding would soon eliminate any genetic differences that might appear. But there is some compelling (albeit indirect) evidence that sympatric speciation can occur.

Speciation in three-spined sticklebacks

The three-spined sticklebacks, freshwater fishes that have been studied by Dolph Schluter (who received his Ph.D. for his work on Darwin's finches with Peter Grant) and his current colleagues in British Columbia, provide an intriguing example that is best explained by sympatric speciation.

They have found:

- Two different species of three-spined sticklebacks in each of five different lakes.
  - a large benthic species with a large mouth that feeds on large prey in the littoral zone
  - a smaller limnetic species with a smaller mouth that feeds on the small plankton in open water.
- DNA analysis indicates that each lake was colonized independently, presumably by a marine ancestor, after the last ice age.
- DNA analysis also shows that the two species in each lake are more closely related to each other than they are to any of the species in the other lakes.
- Nevertheless, the two species in each lake are reproductively isolated; neither mates with the other.
- However, aquarium tests showed that

    - The benthic species from one lake will spawn with the benthic species from the other lakes and likewise the limnetic species from the different lakes will spawn with each other.
    - These benthic and limnetic species even display their mating preferences when presented with sticklebacks from Japanese lakes; that is, a Canadian benthic prefers a Japanese benthic over its close limnetic cousin from its own lake.
- Their conclusion: in each lake, what began as a single population faced such competition for limited resources that
    - **disruptive selection** — competition favoring fishes at either extreme of body size and mouth size over those nearer the mean — coupled with
    - **assortative mating** — each size preferred mates like it

  favored a divergence into two subpopulations exploiting different food in different parts of the lake.
- The fact that this pattern of speciation occurred the same way on three separate occasions suggests strongly that **ecological factors** in a sympatric population can cause speciation.

Sympatric speciation driven by ecological factors may also account for the extraordinary diversity of crustaceans living in the depths of Siberia's Lake Baikal.

How many genes are needed to start down the path to sympatric speciation?

Perhaps not very many. The European corn borer, *Ostrinia nubilalis*, (which despite its common name is a major pest in the U.S. as well) exists as two distinct races designated **Z** and **E**. Both can be found in the same area; that is, they are sympatric. But in the field, they practice assortative mating - only breeding with mates of their own race.

The females of both races synthesize and release a pheromone that is a sex attractant for the males. Both races use the same substance but different isomers of it. Which isomer is produced is under the control of a single enzyme-encoding gene locus. The ability of the males to respond to one isomer or the other is controlled by 2 loci.

The Problem of Clines

There is another possible way for new species to arise in the absence of geographical barriers. If a population ranges over a large area and if the individuals in that population can disperse over only a small portion of this range, then gene flow across these great distances would be reduced. The occurrence of gradual phenotypic (and genotypic) differences in a population across a large geographical area is called a **cline**. Successful interbreeding occurs freely at every point along the cline, but individuals at the ends of the cline may not be able to interbreed. This can be tested in the laboratory.

And, on occasions, it is tested in nature. If a cline bends around so that the ends meet, and the populations reunited at the junction cannot interbreed, then the definition of separate species has been met. Such species are called **ring species** and this type of speciation is called **parapatric speciation**.

Two examples:

1. The Caribbean slipper spurge *Euphorbia tithymaloides*.
Genetic analysis shows that this wildflower originated in Central America where Mexico and Guatemala share a common boundary. From there it spread in two directions

- northeast through the Yucatan peninsula and then island-hopped through Jamaica, the Dominican Republic, Puerto Rico and into the Virgin Islands;
- south through Central America, on through Venezuela, and then north through Barbados and the other islands of the Lesser Antilles finally also reaching the Virgin Islands.

Reunited in the Virgin Islands, the two populations have diverged sufficiently that they retain their distinctive genotypic and phenotypic traits. Ongoing studies will determine to what degree they may be reproductively isolated.

2. The large-blotched salamander *Ensatina eschscholtzii*.

This animal is found in California where it occurs in a number of different subspecies or races. A single subspecies is found in Northern California, and it is thought to be the founder of all the others. Over time that original population spread southward in

two directions:

- down the Sierra Nevada mountains east of the great central San Joaquin Valley and
- down the coast range of mountains west of the valley.

South of the valley, the eastern group has moved west and now meets the western group, closing the ring. Here the two populations fail to interbreed successfully, maintaining their distinct identities. But each subspecies interbreeds in an unbroken chain up the two paths their ancestors took.

Ring species present a difficult problem in assigning species designations. It is easy to say that the populations at the ends of the cline represent separate species, but where did one give rise to the other? At every point along the cline, interbreeding goes on successfully.

The same problem faces paleontologists examining the gradual phenotypic changes seen in an unbroken line of ever-younger fossils from what one presumes to be a single line of descent. If one could resurrect the ancestral species (A) and the descendant species (B) and they could not interbreed, then they meet the definition of separate species. But there was no moment in time when one could say that A became B. So the clines of today are a model in **space** of Darwin's descent with modification occurring over **time**.

Although clines present a problem for classifiers, they are a beautiful demonstration of Darwin's conviction that the accumulation of small inherited differences can lead to the formation of new species.

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18.3: The Evolution of Body Form in Animals

This page explores the paradox of structural diversity and genetic similarity in the animal kingdom, emphasizing the role of mutations in gene regulatory regions that differentiate species, particularly in humans and chimpanzees. Research on a Tc1 mouse carrying human chromosome #21 reveals that most human genes function similarly in mouse cells. While housekeeping and toolkit genes are conserved, species diversity arises from changes in DNA sequences affecting gene expression.

Advances in genetics - especially the sequencing of entire genomes from a wide variety of animals - has revealed an unexpected paradox. While the animal kingdom contains an extraordinary diversity of body types:

- with sizes ranging from the microscopic daphnia (a crustacean) to the great blue whale
- with body plans as diverse as those of Drosophila and humans,

the great structural diversity of animals is **not** reflected in their genetic makeup. Throughout the animal kingdom, one finds thousands of orthologous genes; that is, genes that have similar sequences and encode similar products.

At the level of the cell, and even of tissues, this perhaps should not be surprising. After all, most types of cells - from whatever animal - are quite similar in their structure and function. Thus we would expect that their genes that encode ribosomal proteins, cytochromes, histones, etc., etc. would be similar.

In trying to resolve the paradox that these findings present, it is useful to distinguish between

- "housekeeping" genes - genes that encode proteins (e.g. cytochromes) and RNAs (e.g. ribosomal RNAs) that function in all cells and
- "toolkit" genes
  - genes that control the expression of other genes by encoding transcription factors
  - genes that encode cell-signaling proteins that signal the cell to turn on (or off) a genetic program.

Housekeeping genes generally

- show modest sequence differences from animal to animal. Some of these are neutral in their effect on the gene's function while others are the result of evolutionary adaptation. Both can be used to trace taxonomic relationships.
- have simple control regions, i.e. promoters and enhancers.
- are expressed in all types of cells in the body (almost half of our protein-encoding genes are expressed in every cell - liver, neurons, muscle, etc.).

Toolkit genes generally

- show very slight sequence differences between different animal species in their coding regions (exons) while
- they have very elaborate control regions with many promoter and enhancer sites each of which has a binding site for one or another different transcription factors.

The proteins (transcription factors and cell-signaling molecules) of toolkit genes are identical in whatever cells they are expressed in. However, the function of that protein can vary greatly

- in different tissues of the animal - a phenomenon called mosaic pleiotropy
- at different times in the embryonic development of the animal - a phenomenon called heterochrony

Even such primitive animals as sponges and cnidarians have hundreds of toolkit genes that are clear orthologs to genes of humans. Some examples are genes whose products are involved in cell signaling (e.g., Wnt and β-catenin, Hedgehog, Notch, Receptor Tyrosine Kinases (RTKs), components of JAK/STAT pathways, and Transforming Growth Factor-beta TGF-β receptors).

In fact the sequences of many of these genes from different animal phyla are so similar that they can be interchanged. This similarity can be tested in animals that can be made transgenic. Some examples:

- The mouse gene ***Pax6*** (also known as small eyes [***Sey***] for the mutant phenotype) can substitute for the mutant ***eyeless*** gene in Drosophila while the human *PAX6* gene can restore normal eye development in the mutant small eyes (*Sey*) mouse.
- The mouse homeobox gene *HoxB6* can substitute for the Drosophila homeobox gene *Antennapedia* (*Antp*) and when

introduced into Drosophila give rise to legs in place of antennae just as mutant *Antp* genes do.

Mosaic Pleiotropy

Pleiotropy is the production by a single gene of more than one effect on the phenotype. **Mosaic** refers to the patchy distribution of cells and tissues expressing that phenotype.

An example:

Pitx1 is a

- Homeobox gene (similar to bicoid in Drosophila) with orthologs found in all vertebrates.
- It contains 3 exons that encode a protein of some 283 amino acids (varying slightly in different species) which is
- A transcription factor that regulates the expression of other genes involved in the differentiation and function of
  - the anterior lobe of the pituitary gland (*Pitx1* = "Pituitary homeobox1");
  - jaw development (mutations are associated with cleft palate in mammals);
  - development of the thymus and some types of mechanoreceptors;
  - development of the **hind limbs**.
- Its activity in these regions is controlled by regulatory regions (promoters and/or enhancers) specific to each region (and presumably turned on by other transcription factors in the cells of those regions).

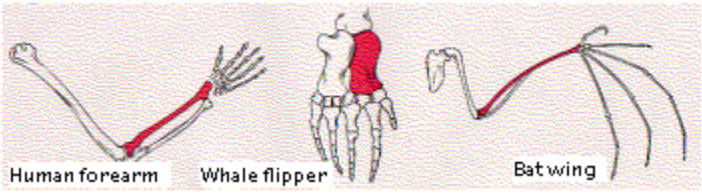


Figure 18.3.1 Forelimbs

When we consider the dramatically-different activities that a given toolkit gene product can perform in different parts of the same animal, it is easier to understand how easy it must be for these same genes to alter the structure of the same body part in different species, e.g., the human arm and the wing of the bat.

Mutations in Regulatory Regions

Not all genes need to be expressed in all cells. In which cells and when a given gene will be expressed is controlled by the interaction of:

- extracellular signals turning on (or off)
- transcription factors, which turn on (or off)
- particular genes

A mutation that would be lethal in the protein coding region of a gene need not be if it occurs in a control region (e.g. promoters and/or enhancers) of that gene. In fact, there is increasing evidence that mutations in control regions have played an important part in evolution. Examples:

- Humans have a gene (*LCT*) encoding lactase; the enzyme that digests lactose (e.g. in milk). In most of the world's people, *LCT* is active in young children but is turned off in adults. However, northern Europeans and three different tribes of African pastoralists, for whom milk remains a part of the adult diet, carry a mutation in the control region of their lactase gene that permits it to be expressed in adults. The mutation is different in each of the 4 cases — examples of convergent evolution.
- There are very few differences in the coding sequences between genes of humans and chimpanzees. However, many of their shared genes differ in their control regions.
- The story of *Prx1*. *Prx1* encodes a transcription factor that is essential for forelimb growth in mammals. When mice have the **enhancer** region of their *Prx1* replaced with the enhancer region of *Prx1* from a bat (whose front limbs are wings), the front legs of resulting mice are 6% longer than normal. Here, then is a morphological change not driven by a change in the Prx1 protein but by a change in the expression of its gene.
- The story of *Pitx1*.

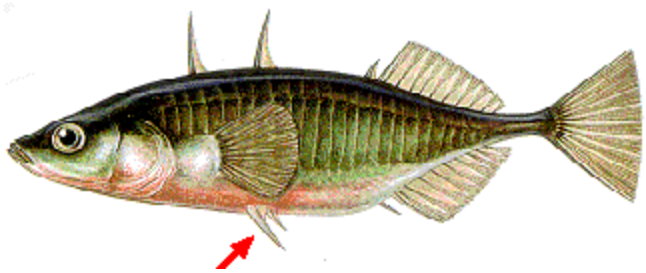


Figure 18.3.2 Stickleback

In a remarkable study of three-spined sticklebacks published in the 15 April 2004 issue of **Nature**, Michael Shapiro, Melissa Marks, Catherine Peichel, and their colleagues report that a mutation in a noncoding region of the *Pitx1* gene accounts for most of the difference in the structure of the pelvic bones of the marine stickleback and its close freshwater cousins.

The marine sticklebacks

- have prominent spines jutting out in their pelvic region (red arrow) as well as the spines along the back (that give the fish its name). These spines may help protect them from being eaten by predators. (Drawing courtesy of the Parks Administration in the Emilia-Romagna region of Italy.)
- express the *Pitx1* gene in various tissues, including
  - thymus
  - mechanoreceptors
  - the pelvic region

The freshwater sticklebacks

- have no or very much smaller spines in their pelvic region
- express the **identical** *Pitx1* gene in all the same tissues **except** those that develop into the pelvic structures
- The reason: a deletion in an enhancer of *Pitx1* responsible for turning on *Pitx1* in the developing pelvic area. (Mice homozygous for a mutation in this control region have deformed hind limbs.)

Here then is a remarkable demonstration of how a single gene mutation can not only be viable but can lead to a major change in phenotype - adaptive evolution. (The changes seem not to have produce true speciation as yet. The marine and freshwater forms can interbreed. In fact, that is how the differences in their hind limbs were found to be primarily due to the expression of *Pitx1*.)

Heterochrony

What toolkit proteins do is governed not only by what tissue they are being produced in but also by when they are produced - a phenomenon called heterochrony.

Examples:

- The transcription factor decapentaplegic (DPP) plays a wide variety of roles in the development of Drosophila from laying the foundation for the future central nervous system in the early embryo to the elaboration of wings, legs, antennae, etc. in the adult.
- The formation of vertebrae. As their name implies, a key feature of all vertebrates is their backbone of vertebrae. However, the number of these can vary greatly. Humans have 33 while snakes can have several hundred. However, the toolkit genes (e.g., Wnt, Notch, FGF-β) responsible for forming vertebrae appear to be the same for all. What makes the difference is the timing (rate) at which pulses of these proteins are produced relative to the rate at which the embryo grows.

The Tc1 Mouse

So the evidence is increasing that what makes the difference between a human and a chimpanzee (or any other pair of animals) is in large measure

- not a matter of their inheritance of different genes and their encoded proteins and RNAs but

- their inheritance of mutations in the control regions - promoters and enhancers - that regulate **where** and **when** these genes will be expressed.

A vivid example of this is the work reported by Wilson *et al*. in the 17 October 2008 issue of **Science**. Their experimental material was liver cells (hepatocytes) taken from

- normal humans
- normal mice
- the Tc1 mouse

The Tc1 mouse is more than simply transgenic, it carries in most of its cells a human chromosome #21. This small chromosome is the one that, when present in a triple dose (trisomy 21), produces Down syndrome in humans. Mice have a similar chromosome that is designated #16.

The question that this remarkable animal could answer: will the genes on human chromosome #21 (105 of them) when present in a mouse nucleus and surrounded by mouse transcription factors and signaling pathways respond as the mouse #16 does or as #21 does in human liver cells or something quite different from either?

The answer turned out that the #21 responded pretty much as it does in its normal human cellular environment.

One line of evidence

Several transcription factors turn on gene activity in liver cells. As seems to be the case with all transcription factors, the human and mouse versions are close orthologs (95% identical in sequence). Using ChIP analysis, they found that the mouse transcription factors bound to sites along the human chromosome much as the human transcription factors do. (Chromosome #21 does not encode any transcription factors, so all those available in the mouse nucleus were of mouse origin.)

Tissue	Chromosome	Transcription Factors (TFs)	Sites Bound by TFs	Gene Expression
human liver cells	#21	human TFs	human pattern	human pattern
Tc1 liver cells	#21	mouse TFs	human pattern	human pattern
	#16	mouse TFs	mouse pattern	mouse pattern
normal mouse liver cells	#16	mouse TFs	mouse pattern	mouse pattern

A second line of evidence: gene expression

These workers also examined the pattern of gene expression; that is, the production of messenger RNAs, in the various combinations. They did this using **microarrays.** The human genes expressed on chromosome #21 by mouse transcription factors in the Tc1 mouse cells were mostly the same as those turned on by human transcription factors in human cells.

The Bottom Line

All these lines of evidence point to the following:

Throughout the animal kingdom,

- Genes encoding "housekeeping" proteins (histones, enzymes, etc., etc.) are remarkably conserved; that is, their products vary only slightly - certainly not enough to account for the vast range of bodies seen in animals from sea anemones to humans.
- Genes encoding "toolkit" proteins (transcription factors, cell signaling molecules) are also highly conserved (even more so).
- So the key to the fabulous diversity in the animal kingdom must lie in what evolution has done to the DNA sequences that control where and when genes are expressed in the developing animal.

So after years of looking for and sequencing open reading frames, the task now will be to analyze the sequence differences - arisen by mutation and evolution - in the **intergenic** regions that serve as control regions of those genes. An early result of genome analysis: during the radiation of the various mammalian orders, **enhancers** have diversified (evolved) much more rapidly

than have promoters and their associated protein-encoding genes.

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18.4: Recapitulation

The embryonic development of all vertebrates shows remarkable similarities as you can see from these drawings (supplied by Open Court Publishing Company). The drawings in the top row are of the embryonic stage called the **pharyngula**. At this stage ("**I**") they all contain a notochord, dorsal hollow nerve cord, a tail extending behind the anus, and a series of **paired branchial grooves.**

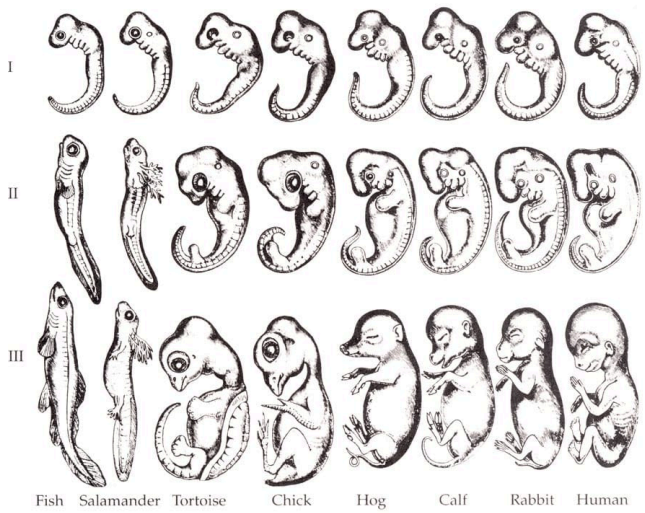


Figure 18.4.1 George Romanes's 1892 copy of Ernst Haeckel's controversial embryo drawings. (Public Domain).

The branchial grooves are matched on the inside by a series of paired **gill pouches**. In fishes, the pouches and grooves eventually meet and form the **gill slits**, which allow water to pass from the pharynx over the gills and out the body. In the other vertebrates shown here, the grooves and pouches disappear. In humans, the chief trace of their existence is the eustachian tube and auditory canal which (interrupted only by the eardrum) connect the pharynx with the outside of the head.

Anatomical Recapitulation

The idea that embryonic development repeats that of one's ancestors is called **recapitulation**. It is often expressed as "ontogeny recapitulates phylogeny"; that is, embryonic development (ontogeny) repeats phylogeny (the genealogy of the species). This is a distortion of the truth and implies that early in our embryonic development we go through a fishlike stage. We do not. Rather, we pass through some (not all) of the embryonic stages that our ancestors passed through. Therefore, we find that the more distantly related two vertebrates are, the shorter the period during which they pass through similar embryonic stages (fish and human) and vice versa (fish and salamander).

We should also keep in mind that embryonic development prior to the pharyngula (stage **I**) may also be very different in the different groups. For example, while the pharyngulas of the human and the salamander look quite similar, their earlier development, starting with their fertilized eggs, are very different. The idea that "ontogeny recapitulates phylogeny" was proposed over a century ago by the biologist Ernst Haeckel. He also made the drawings on which the drawings above are based. Periodically, people rediscover that in making them, he altered certain details to emphasize his theory. Though they are schematic, the story they illustrate here has stood the test of time.

Another Example

Fossil evidence suggests that the ancestors of the insects had a pair of legs on each of their body segments. In this, they resembled today's millipedes (which may represent a second line of descent from these early forms). In any case, during embryonic development of insects, limb buds appear on the abdomen just as they must have in their multilegged ancestors. But

by the time the larva hatches, only the six legs on the thorax (color) remain. The ones in the head become mouth parts. The ones in the abdomen disappear. Both these changes appear to be controlled by the expression of HOX genes.

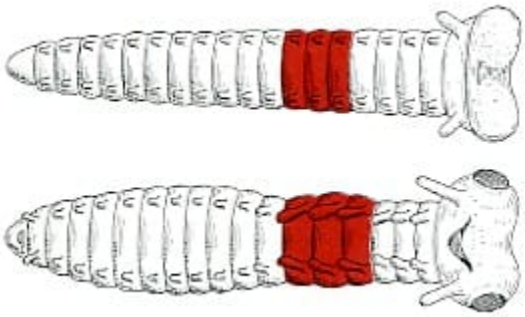


Figure 18.4.2 Limb buds

Biochemical Recapitulation

Fish excrete a large part of their waste nitrogen as ammonia (NH_3), while amphibians have the less toxic **urea** as their nitrogenous waste. Actually, the fish-like tadpole excretes ammonia until it undergoes metamorphosis into the adult frog. Only then does its chief nitrogenous waste become urea.

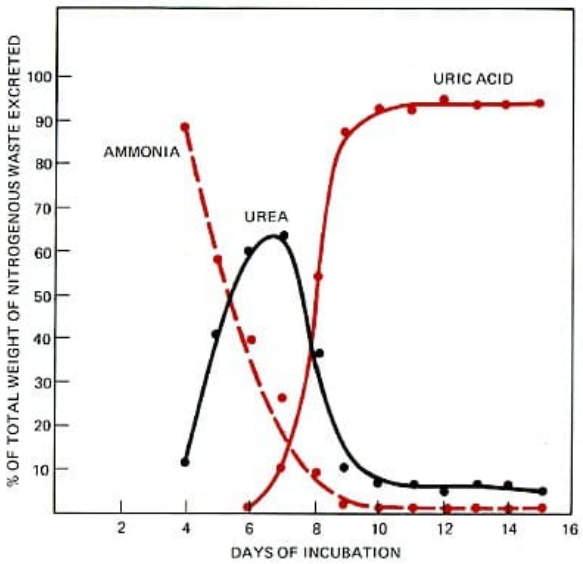


Figure 18.4.3 Biochem recapitulation

As for reptiles and birds, they convert their waste nitrogen compounds into the almost insoluble **uric acid**. During its development within the egg, however, the chicken embryo first passes through a stage of ammonia excretion followed by a period in which it excretes urea before finally turning to uric acid.

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18.5: Mutation and Evolution

Mutations are the raw materials of evolution. Evolution absolutely depends on mutations because this is the only way that new alleles and new regulatory regions are created. However, this seems paradoxical because most mutations that we observe are harmful (e.g., many missense mutations) or, at best, neutral, For example, "silent" mutations encoding the same amino acid. Also, many of the mutations in the vast amounts of DNA that lie between genes. Morevoer, most mutations in genes affect a single protein product (or a small set of related proteins produced by alternative splicing of a single gene transcript) while much evolutionary change involves myriad structural and functional changes in the phenotype.

So how can the small changes in genes caused by mutations, especially single-base substitutions ("point mutations"), lead to the large changes that distinguish one species from another? These questions have, as yet, only tentative answers.

One Solution: Duplication of Genes and Genomes

Mutations that would be harmful in a single pair of genes can be tolerated if those genes have first been duplicated. Gene duplication in a diploid organism provides a second pair of genes so that one pair can be safely mutated and tested in various combinations while the essential functions of the parent pair are kept intact.

Possible benefits:

- Over time, one of the duplicates can acquire a new function. This can provide the basis for adaptive evolution.
- But even while two paralogous genes are still similar in sequence and function, their existence provides **redundancy** ("belt and suspenders"). This may be a major reason why knocking out genes in yeast, "knockout mice", etc. so often has such a mild effect on the phenotype. The function of the knocked out gene can be taken over by a paralog.
- After gene duplication, random loss of these genes at a later time in one group of descendants different from the loss in another group could provide a barrier (a "post-zygotic isolating mechanism") to their interbreeding. Such a barrier could cause speciation: the evolution of two different species from a single ancestral species.

Evidence:

- Paralogous genes. Genes in one species that have arisen by duplication of an ancestral gene. Example: genes encoding olfactory receptors.
- Duplication of the entire genome. Examples:
  - Polyploid angiosperms.
  - Genome analysis of three ascomycetes show that early in the evolution of the budding yeast, *Saccharomyces cerevisiae*, its entire genome was duplicated. Each chromosome of the other ascomycetes contains stretches of genes whose orthologs are distributed over **two** *Saccharomyces cerevisiae* chromosomes.
  - There is also evidence that vertebrate evolution has involved at least two duplications of the entire genome. Example: both the invertebrate Drosophila and the invertebrate chordate Amphioxus contain a single **HOX gene cluster** while mice and humans have **four**.

A Second Solution: Mutations in Regulatory Regions

Not all genes are expressed in all cells. In which cells and when a given gene will be expressed is controlled by the interaction of (1) extracellular signals turning on (or off),(2) transcription factors, which turn on (or off), and (3) particular genes. A mutation that would be lethal in the protein coding region of a gene need not be if it occurs in a control region (e.g. promoters and/or enhancers) of that gene. In fact, there is increasing evidence that mutations in control regions have played an important part in evolution. Examples:

- Humans have a gene (*LCT*) encoding lactase; the enzyme that digests lactose (e.g. in milk). In most of the world's people, *LCT* is active in young children but is turned off in adults. However, northern Europeans and three different tribes of African pastoralists, for whom milk remains a part of the adult diet, carry a mutation in the control region of their lactase gene that permits it to be expressed in adults. The mutation is different in each of the 4 cases examples of convergent evolution.
- There are very few differences in the coding sequences between genes of humans and chimpanzees. However, many of their

shared genes differ in their control regions.
- The story of *Prx1*. *Prx1* encodes a transcription factor that is essential for forelimb growth in mammals. When mice have the **enhancer** region of their *Prx1* replaced with the enhancer region of *Prx1* from a bat (whose front limbs are wings), the front legs of resulting mice are 6% longer than normal. Here, then is a morphological change not driven by a change in the Prx1 protein but by a change in the expression of its gene.
- The story of *Pitx1*
- The story of *Style2.1* in the domestic tomato

A Third Solution?

Another theoretically-possible way by which a point mutation might give rise to a new gene is if the point mutation in a previously noncoding section of DNA converts a triplet of nucleotides into ATG thus creating a new open reading frame (ORF). It is increasingly evident that much of noncoding DNA is transcribed into a heterogeneous collection of RNAs. Transcription of DNA with its newly-acquired ATG codon would produce an RNA molecule with a translation start codon (AUG). Translation of this RNA would create a protein that most likely would be useless, perhaps even harmful but might, on rare occasions, provide the starting point for the acquisition of a new useful gene.

Large Changes in Phenotype can come from small changes in Genotype

Selector Genes

The building of an organ requires the coordinated activity of many genes. However, these are often organized in hierarchies so that "upstream genes" regulate the activity of "downstream genes". The closer you get to the top with a mutation, the greater the changes affected downstream.

Follow these links to see examples of the influence of "master" (selector) genes on the phenotype.

- Embryonic Development: Getting Started (especially the story of *bicoid* and *nanos*)
- Organizing the Embryo: The Central Nervous System Organizing the Embryo: Segmentation (more on *bicoid* and *nanos*)
- Embryonic Development: Putting on the finishing touches (especially the discussion of homeobox genes)

The Story of Pitx1

Pitx1 is homeobox gene (similar to bicoid in Drosophila) with orthologs found in all vertebrates. It contains 3 exons that encode a protein of some 283 amino acids (varying slightly in different species) which is a transcription factor that regulates the expression of other genes involved in the differentiation and function of multiple features including:

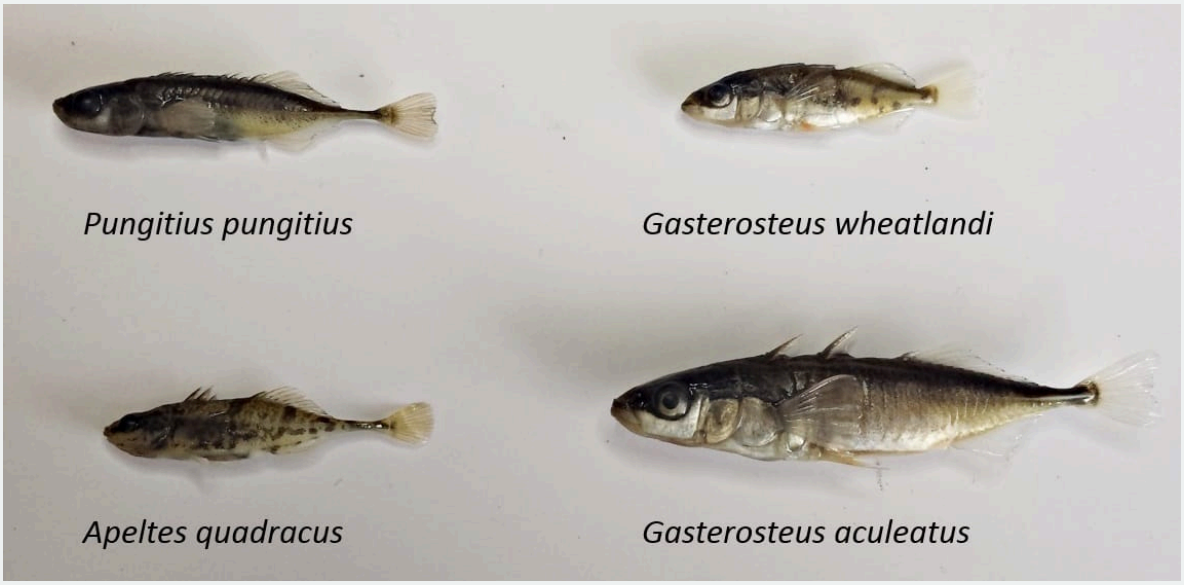
1. the anterior lobe of the pituitary gland (*Pitx1* = "Pituitary homeobox1");
2. jaw development (mutations are associated with cleft palate);
3. development of the thymus and some types of mechanoreceptors;
4. development of the **hind limbs**.

Its activity in these regions is controlled by regulatory regions (promoters and/or enhancers) specific to each region (and presumably turned on by other transcription factors in the cells of those regions).

Pitx1 is an essential gene. Mutations in the **coding** regions are lethal when homozygous (shown in mice). However, mutations in **noncoding** regions need not be. All vertebrates have a pelvic girdle with associated bones which make up the pelvic fins of fishes and the hind legs of the tetrapods. *Pitx1* is needed by them all for the proper development of these structures (as well as the other functions of *Pitx1*).

In a remarkable study of three-spined sticklebacks published in the 15 April 2004 issue of **Nature**, Michael Shapiro, Melissa Marks, Catherine Peichel, and their colleagues report that a mutation in a noncoding region of the *Pitx1* gene accounts for most of the difference in the structure of the pelvic bones of the marine stickleback and its close freshwater cousins.

The marine sticklebacks have prominent spines jutting out in their pelvic region (red arrow) as well as the spines along the back (that give the fish its name). These spines may help protect them from being eaten by predators. (Drawing courtesy of the Parks Administration in the Emilia-Romagna region of Italy.) The also express the *Pitx1* gene in various tissues, including thymus, mechanoreceptors, and the pelvic region.



The four species of stickleback that inhabit the Atlantic coast of North America. These species are sympatric between Newfoundland, Canada and Long Island, New York, United States. Image used iwth permission (CC BY-SA 3.0; Ghegeman).

The freshwater sticklebacks have no — or very much smaller — spines in their pelvic region. They express the **identical** *Pitx1* gene in all the same tissues **except** those that develop into the pelvic structures. The reason: a mutation in an enhancer upstream of the *Pitx1* exons. The unmutated enhancer turns on *Pitx1* in the developing pelvic area. (Mice homozygous for a mutation in this control region have deformed hind limbs.)

Here then is a remarkable demonstration of how a single gene mutation can not only be viable but can lead to a major change in phenotype - adaptive evolution. (The changes seem not to have produce true speciation as yet. The marine and freshwater forms can interbreed. In fact, that is how the differences in their hind limbs were found to be primarily due to the expression of *Pitx1*.)

A survey of 21 different populations of sticklebacks - both freshwater and marine - from different regions of North America, Europe, and Japan has revealed a pattern of consistent genetic differences that distinguish the freshwater from the marine forms. However, only 17% of the distinguishing mutations were found in exons that alter the amino acid sequence of the encoded proteins. All the rest were "silent" and most, 41% or more, of these occurred in intergenic regions. These results further demonstrate the importance of mutations in regulatory regions - promoters and enhancers - in the evolution of adaptive phenotypes.

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18.6: The Hardy-Weinberg Equilibrium

If two individuals mate that are heterozygous (e.g., **Bb**) for a trait, we find that

- 25% of their offspring are homozygous for the dominant allele (**BB**)
- 50% are heterozygous like their parents (**Bb**)
- 25% are homozygous for the recessive allele (**bb**) and thus, unlike their parents, express the recessive phenotype.

This is what Mendel found when he crossed monohybrids. It occurs because meiosis separates the two alleles of each heterozygous parent so that 50% of the gametes will carry one allele and 50% the other and when the gametes are brought together at random, each **B** (or **b**)-carrying egg will have a 1 in 2 probability of being fertilized by a sperm carrying **B** (or **b**). (Left table)

Results of random union of the two gametes produced by two individuals, each heterozygous for a given trait. As a result of meiosis, half the gametes produced by each parent with carry allele B; the other half allele b.			**Results of random union of the gametes produced by an entire population with a gene pool containing 80% B and 20% b.**		
	0.5 **B**	0.5 **b**		0.8 **B**	0.2 **b**
0.5 **B**	0.25 **BB**	0.25 **Bb**	0.8 **B**	0.64 **BB**	0.16 **Bb**
0.5 **b**	0.25 **Bb**	0.25 **bb**	0.2 **b**	0.16 **Bb**	0.04 **bb**

However, the frequency of two alleles in an **entire population** of organisms is unlikely to be exactly the same. Let us take as a hypothetical case, a population of hamsters in which 80% of all the gametes in the population carry a dominant allele for black coat (**B**) and 20% carry the recessive allele for gray coat (**b**).

Random union of these gametes (right table) will produce a generation:

- 64% homozygous for **BB** (0.8 x 0.8 = 0.64)
- 32% **Bb** heterozygotes (0.8 x 0.2 x 2 = 0.32)
- 4% homozygous (**bb**) for gray coat (0.2 x 0.2 = 0.04)

So 96% of this generation will have black coats; only 4% gray coats.

Will gray coated hamsters eventually disappear? No. Let's see why not.

- All the gametes formed by **BB** hamsters will contain allele **B** as will one-half the gametes formed by heterozygous (**Bb**) hamsters.
- So, 80% (0.64 + .5*0.32) of the pool of gametes formed by this generation with contain **B**.
- All the gametes of the gray (**bb**) hamsters (4%) will contain **b** but one-half of the gametes of the heterozygous hamsters will as well.
- So 20% (0.04 + .5*0.32) of the gametes will contain **b**.

So we have duplicated the initial situation exactly. The proportion of allele **b** in the population has remained the same. The heterozygous hamsters ensure that each generation will contain 4% gray hamsters. Now let us look at an algebraic analysis of the same problem using the expansion of the binomial (**p**+**q**)^2.

$$(p+q)^2 = p^2 + 2pq + q^2 \tag{18.6.1}$$

The total number of genes in a population is its *gene pool*.

- Let p represent the frequency of one gene in the pool and q the frequency of its single allele.
- So, $p + q = 1$
  - p^2 = the fraction of the population homozygous for p
  - q^2 = the fraction homozygous for q
  - $2pq$ = the fraction of heterozygotes

- In our example, **p** = 0.8, **q** = 0.2, and thus

$$(0.8 + 0.2)^2 = (0.8)^2 + 2(0.8)(0.2) + (0.2)^2 = 064 + 0.32 + 0.04 \quad (18.6.2)$$

The algebraic method enables us to work backward as well as forward. In fact, because we chose to make **B** fully dominant, the only way that the frequency of **B** and **b** in the gene pool could be known is by determining the frequency of the recessive phenotype (gray) and computing from it the value of **q**.

q^2 = 0.04, so **q** = 0.2, the frequency of the **b** allele in the gene pool. Since **p** + **q** = 1, **p** = 0.8 and allele **B** makes up 80% of the gene pool. Because **B** is completely dominant over **b**, we cannot distinguish the **Bb** hamsters from the **BB** ones by their phenotype. But substituting in the middle term (**2pq**) of the expansion gives the percentage of heterozygous hamsters. **2pq** = (2)(0.8)(0.2) = 0.32

So, recessive genes do not tend to be lost from a population no matter how small their representation.

Hardy-Weinberg law

So long as certain conditions are met (discussed below), **gene frequencies** and **genotype ratios** in a randomly-breeding population remain constant from generation to generation. This is known as the Hardy-Weinberg law.

The Hardy-Weinberg law is named in honor of the two men who first realized the significance of the binomial expansion to population genetics and hence to evolution. Evolution involves changes in the gene pool. A population in Hardy-Weinberg equilibrium shows no change. What the law tells us is that populations are able to maintain a reservoir of variability so that if future conditions require it, the gene pool can change. If recessive alleles were continually tending to disappear, the population would soon become homozygous. Under Hardy-Weinberg conditions, genes that have no present selective value will nonetheless be retained.

When the Hardy-Weinberg Law Fails

To see what forces lead to evolutionary change, we must examine the circumstances in which the Hardy-Weinberg law may fail to apply. There are five:

1. mutation
2. gene flow
3. genetic drift
4. nonrandom mating
5. natural selection

Mutation

The frequency of gene **B** and its allele **b** will not remain in Hardy-Weinberg equilibrium if the rate of mutation of **B** -> **b** (or vice versa) changes. By itself, this type of mutation probably plays only a minor role in evolution; the rates are simply too low. However, gene (and whole genome) duplication - a form of mutation - probably has played a major role in evolution. In any case, evolution absolutely depends on mutations because this is the only way that new alleles are created. After being shuffled in various combinations with the rest of the gene pool, these provide the raw material on which natural selection can act.

Gene Flow

Many species are made up of local populations whose members tend to breed within the group. Each local population can develop a gene pool distinct from that of other local populations. However, members of one population may breed with occasional immigrants from an adjacent population of the same species. This can introduce new genes or alter existing gene frequencies in the residents.

In many plants and some animals, gene flow can occur not only between subpopulations of the same species but also between different (but still related) species. This is called **hybridization**. If the hybrids later breed with one of the parental types, new

genes are passed into the gene pool of that parent population. This process is called **introgression**. It is simply gene flow between species rather than within them.

Comparison of the genomes of contemporary humans with the genome recovered from Neanderthal remains shows that from 1–3% of our genes were acquired by introgression following mating between members of the two populations tens of thousands of years ago.

Whether within a species or between species, gene flow increases the variability of the gene pool.

Genetic Drift

As we have seen, interbreeding often is limited to the members of local populations. If the population is small, Hardy-Weinberg may be violated. Chance alone may eliminate certain members out of proportion to their numbers in the population. In such cases, the frequency of an allele may begin to drift toward higher or lower values. Ultimately, the allele may represent 100% of the gene pool or, just as likely, disappear from it.

Drift produces evolutionary change, but there is no guarantee that the new population will be more fit than the original one. Evolution by drift is aimless, not adaptive.

Nonrandom Mating

One of the cornerstones of the Hardy-Weinberg equilibrium is that mating in the population must be random. If individuals (usually females) are choosy in their selection of mates, the gene frequencies may become altered. Darwin called this **sexual selection**.

Nonrandom mating seems to be quite common. Breeding territories, courtship displays, "pecking orders" can all lead to it. In each case certain individuals do not get to make their proportionate contribution to the next generation.

Assortative mating

Humans seldom mate at random preferring phenotypes like themselves (e.g., size, age, ethnicity). This is called *assortative mating*. Marriage between close relatives is a special case of assortative mating. The closer the kinship, the more alleles shared and the greater the degree of **inbreeding**. Inbreeding can alter the gene pool. This is because it predisposes to **homozygosity**. Potentially harmful recessive alleles — invisible in the parents — become exposed to the forces of natural selection in the children.



Figure 18.6.1: Assortative mating. (Drawing by Koren © 1977 The New Yorker Magazine, Inc.)

It turns out that many species - plants as well as animals - have mechanisms be which they avoid inbreeding. Examples:

- Link to discussion of self-incompatibility in plants.
- Male mice use olfactory cues to discriminate against close relatives when selecting mates. The preference is learned in infancy - an example of imprinting. The distinguishing odors are controlled by the MHC alleles of the mice and are detected by the vomeronasal organ (VNO).

Natural Selection

If individuals having certain genes are better able to produce mature offspring than those without them, the frequency of those genes will increase. This is simply expressing Darwin's natural selection in terms of alterations in the gene pool. (Darwin knew nothing of genes.) Natural selection results from *differential mortality* and/or *differential fecundity*.

Mortality Selection

Certain genotypes are less successful than others in surviving through to the end of their reproductive period. The evolutionary impact of mortality selection can be felt anytime from the formation of a new zygote to the end (if there is one) of the organism's period of fertility. Mortality selection is simply another way of describing Darwin's criteria of fitness: **survival**.

Fecundity Selection

Certain phenotypes (thus genotypes) may make a disproportionate contribution to the gene pool of the next generation by producing a disproportionate number of young. Such fecundity selection is another way of describing another criterion of fitness described by Darwin: **family size**. In each of these examples of natural selection, certain phenotypes are better able than others to contribute their genes to the next generation. Thus, by Darwin's standards, they are more **fit**. The outcome is a gradual change in the gene frequencies in that population.

Calculating the Effect of Natural Selection on Gene Frequencies

The effect of natural selection on gene frequencies can be quantified. Let us assume a population containing

- 36% homozygous dominants (**AA**)
- 48% heterozygotes (**Aa**) and
- 16% homozygous recessives (**aa**)

The gene frequencies in this population are $p = 0.6$ and $q = 0.4$. The heterozygotes are just as successful at reproducing themselves as the homozygous dominants, but the homozygous recessives are only 80% as successful. That is, for every 100 **AA** (or **Aa**) individuals that reproduce successfully only 80 of the **aa** individuals succeed in doing so. The *fitness* (w) of the recessive phenotype is thus 80% or 0.8.

Their relative disadvantage can also be expressed as a *selection coefficient*, s, where

$$s = 1 - w \tag{18.6.3}$$

In this case,

$$s = 1 - 0.8 = 0.2. \tag{18.6.4}$$

The change in frequency of the dominant allele (Δp) after one generation is expressed by the equation

$$\Delta p = \frac{s p_0 q_0^2}{1 - s q_0^2} \tag{18.6.5}$$

where p_0 and q_0 are the initial frequencies of the dominant and recessive alleles respectively. Substituting, we get

$$\Delta p = \frac{(0.2)(0.6)(0.4)^2}{1-(0.2)(0.4)^2} \tag{18.6.6}$$

$$= \frac{0.019}{0.968} \tag{18.6.7}$$

$$= 0.02 \tag{18.6.8}$$

So, in one generation, the frequency of allele **A** rises from its initial value of 0.6 to 0.62 and that of allele **a** declines from 0.4 to 0.38 ($q = 1 - p$).

The new equilibrium produces a population of

- 38.4% homozygous dominants (an increase of 2.4%) ($\mathbf{p^2}$ = 0.384)
- 47.1% heterozygotes (a decline of 0.9%)(**2pq** = 0.471) and
- 14.4% homozygous recessives (a decline of 1.6%)($\mathbf{q^2}$ = 0.144)

If the fitness of the homozygous recessives continues unchanged, the calculations can be reiterated for any number of generations. If you do so, you will find that although the frequency of the recessive genotype declines, the **rate** at which **a** is removed from the gene pool declines; that is, the process becomes less efficient at purging allele **a**. This is because when present in the heterozygote, **a** is protected from the effects of selection.

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18.7: Polymorphisms

A polymorphism is a genetic variant that appears in at least 1% of a population. (e.g., the human ABO blood groups, the human Rh factor, and the human major histocompatibility complex). By setting the cutoff at 1%, it excludes spontaneous mutations that may have occurred in - and spread through the descendants of - a single family.

Protein Polymorphisms

All the examples above are of the protein products of alleles. These can be identified by serology - that is, using antibodies to detect the different versions of the protein. (Antibodies caused the clumping of the red blood cells in this test) and electrophoresis - if amino acid changes in the protein alter its net electrical charge, it will migrate more or less rapidly in an electrical field. Enzymes are frequently polymorphic. A population may contain two or more variants of an enzyme encoded by a single locus. The variants differ slightly in their amino acid sequence and often this causes them to migrate differently under electrophoresis. By treating the gel with the substrate for the enzyme, its presence can be visualized.

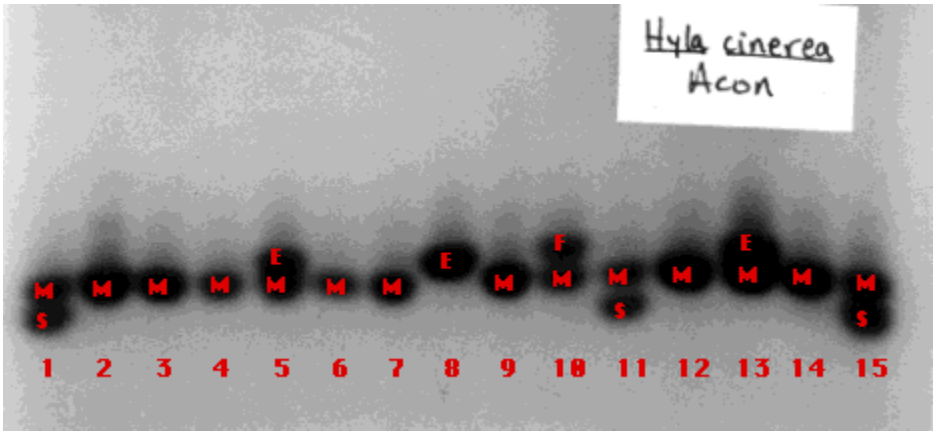


Figure 18.7.1 Electrophoresis of green treefog tissues. Courtesy of Susan McAlpine. The 4 alleles can be distinguished by the speed with which their protein product migrates: Fast (**F**), moderately fast (**E**), medium (**M**), and slow (**S**)

Electrophoresis of tissue extracts from 15 different green treefrogs (***Hyla cinerea***) reveals 4 allelic versions of the enzyme **aconitase** (one of the enzymes of the citric acid cycle). The results:

- Eight frogs (#2, 3, 4, 6, 7, 9, 12, and 14) were homozygous for allele **M**.
- Frog #8 was homozygous for allele **E**.
- Three frogs (#1, 11, 15) are **heterozygous** for the **M** and **S** alleles.
- Two (#5, 13) were heterozygous for **M** and **E**.
- Frog #10 was heterozygous for **M** and **F**.

Electrophoretic variants of an enzyme occurring in a population are called *allozymes*.

Restriction Fragment Length Polymorphisms (RFLPs)

Proteins are gene products and so polymorphic versions are simply reflections of allelic differences in the gene; that is, allelic differences in DNA. Often these changes create new - or abolish old - sites for restriction enzymes to cut the DNA. Digestion with the enzyme then produces DNA fragments of a different length. These can be detected by electrophoresis. Most* RFLPs are created by a change in a single nucleotide in the gene, and so these are called single *nucleotide polymorphisms* (SNPs).

Single Nucleotide Polymorphisms (SNPs)

Developments in DNA sequencing now make it easy to look for allelic versions of a gene by sequencing samples of the gene

taken from different members of a population (or from a heterozygous individual). Alleles whose sequence reveals only a single changed nucleotide are called single nucleotide polymorphisms or **SNPs**. SNPs can occur in noncoding parts of the gene so they would not be seen in the protein product. They might not alter the cutting site for any known restriction enzymes so they would not be seen by RFLP analysis. As of October 2005, over one million SNPs had been identified across the human genome.

Copy Number Polymorphisms (CNPs)

Genetic analysis (using DNA chips and FISH) has revealed another class of human polymorphisms. These **copy number polymorphisms** are large (thousands of base pairs) duplications or deletions that are found in some people but not in others. On average, one person differs from another by 11 of these. One or more have been found on most chromosomes, and the list is probably incomplete. While most of this DNA is non-coding, functional genes are embedded in some of it. Example: *AMY1*, the gene encoding salivary amylase, an enzyme that digests starch. Humans vary in the number of copies of *AMY1* in their genome.

- Populations whose diet is rich in starches (e.g., many Americans, Japanese) have an average of 7 copies of the gene.
- Populations with low-starch diets (e.g., nomadic tribes in Siberia whose diet is dominated by dairy products and fish) average only 5 copies.

In the case of *AMY1*, the more copies present, the more enzyme that is produced. How a person adapts to a change in gene number for autosomal genes is unknown (in contrast to the way that human females adjust the activity of the genes on their two X chromosomes to match that of males with their solitary X chromosome).

How are polymorphisms useful?

Polymorphism analysis is in widespread use. In tissue typing, it is use to find the best match between the donor, e.g., of a kidney, and the recipient. It is used to find disease genes (e.g., the gene for Huntington's disease was located when the presence of the disease was found to be linked to a RFLP whose location on the chromosome was known). In population studies, it is used to assess the degree of genetic diversity in a population, including:

- The McAlpine study, which produced the photo above, found that the heterozygous frogs were more successful breeders than homozygous ones.
- A search for polymorphisms in elephant seals and cheetahs has revealed that they have few or none.
- Determining whether two populations represent separate species or races of the same species. This is often critical to applying laws protecting endangered species.

Tracking migration patterns of a species (e.g., whales).

How do polymorphisms arise and persist?

They arise by **mutation**. But what keeps them in the population? Several factors may maintain polymorphism in a population.

- **Founder Effect:** If a population began with a few individuals — one or more of whom carried a particular allele — that allele may come to be represented in many of the descendants. In the 1680s Ariaantje and Gerrit Jansz emigrated from Holland to South Africa, one of them bringing along an allele for the mild metabolic disease **porphyria**. Today more than 30000 South Africans carry this allele and, in every case examined, can trace it back to this couple — a remarkable example of the founder effect.
- **Genetic Drift:** An allele may increase - or decrease - in frequency simply through chance. Not every member of the population will become a parent and not every set of parents will produce the same number of offspring. The effect, called random genetic drift, is particularly strong in small populations (e.g., 100 breeding pairs or fewer) and when the allele is neutral; that is, is neither helpful nor deleterious

Eventually the entire population may become homozygous for the allele or - equally likely - the allele may disappear. Before either of these fates occurs, the allele represents a polymorphism.

Two examples of **reduced** polymorphism because of genetic drift:

- By 1900, hunting of the northern **elephant seal** off the Pacific coast had reduced its population to only 20 survivors. Since hunting ended, the population has rebounded from this **population bottleneck** to some 100,000 animals today. However,

these animals are homozygous at every one of the gene loci that have been examined.

- **Cheetahs**, the fastest of the land animals, seem to have passed through a similar period of small population size with its accompanying genetic drift. Examination of 52 different loci has failed to reveal any polymorphisms; that is, these animals are homozygous at all 52 loci. The lack of genetic variability is so profound that cheetahs will accept skin grafts from each other just as identical twins (and inbred mouse strains) do. Whether a population with such little genetic diversity can continue to adapt to a changing environment remains to be seen.

Natural Selection

Copy Number Polymorphisms

The varying number of copies of the *AMY1* gene in different human populations appears to have arisen from the evolutionary pressure of the differences in the starch content of their diet.

Balanced Polymorphism

In regions of the world (e.g., parts of Africa) where malaria caused by *Plasmodium falciparum* is common, the allele for sickle-cell hemoglobin is also common. This is because children who inherit one gene for the "normal" beta chain of hemoglobin and one sickle gene are more likely to survive than either homozygote. Children homozygous for the sickle allele die young from sickle-cell disease but children homozygous for the "normal" beta chain are more susceptible to illness and death from falciparum malaria than are heterozygotes. Hence the relatively high frequency of the allele in malarial regions. When natural selection favors heterozygotes over both homozygotes, the result is **balanced polymorphism**. It accounts for the persistence of an allele even though it is deleterious when homozygous.

Another example: prion proteins

All human populations are polymorphic for the prion protein PrP^C. It is encoded by the prion protein gene (*PRNP*). Two of the alleles have different codons at position 129 - one encoding **methionine**; the other **valine**. Homozygosity for **either allele** increases the susceptibility to prion diseases. People who are heterozygous are more resistant. A study of elderly women who had survived the kuru epidemic of the first half of the 20th century (eating the tissues of the deceased was banned in 1950) showed that 76.7% of them were heterozygotes. This table compares the gene frequencies in this population as well as in a population that never practiced mortuary feasts.

Table 1: M is the allele encoding the methionine; V the allele encoding valine.

	MM	*MV*	*VV*
Survivors	0.133	0.767	0.100
Unexposed	0.221	0.514	0.264

A quick calculation will show that the gene pool of the exposed women deviates widely from what would be found if the population were in Hardy-Weinberg equilibrium. In this case, strong mortality selection is the cause. The gene pool of the unexposed population is close to being in Hardy-Weinberg equilibrium. Here, again, natural selection has favored heterozygotes over both homozygotes (and led to the speculation that cannibalism may have been common earlier in human history).

Natural vs. Sexual Selection: Balanced polymorphism in Soay sheep

Hirta is a tiny island in the North Atlantic 100 miles off the northwest coast of Scotland. In 1932 a small (107) population of domestic sheep (*Ovis aries*) was introduced onto the island from the neighboring island of Soay. Since then these sheep have been allowed to run wild and, since 1985, have been intensively studied. The sheep have horns and, in males, these play an important role in competition for females. The size of the horns is strongly influenced by a single gene locus, *RXFP2*, with two alleles: Ho^+ and Ho^P.

- Homozygous $Ho^{+}Ho^{+}$ males have the largest horns and sire more offspring but have reduced survival.
- Homozygous $Ho^{P}Ho^{P}$ males have smaller horns (sometime even vestigial horns called scurs). These males have less success in mating but have increased survival.
- Heterozygous $Ho^{+}Ho^{P}$ males are almost as successful at mating as $Ho^{+}Ho^{+}$ males and survive almost as well as $Ho^{P}Ho^{P}$ males. On balance, then, the heterozygotes have greater overall fitness than either homozygotes — another example of **balanced polymorphism**. It arises as a trade-off between the opposing effects of **natural selection** (survival) and **sexual selection** (reproductive success) on a single gene locus.

You can read about these findings in Johnston, Susan. E., *et al.*, *Nature* **502**, 93–95, 3 October 2013.

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18.8: Kin Selection

In the discussion of natural selection, the emphasis was on how natural selection works on individuals to favor the more fit and disfavor the less fit in a population. The emphasis was on the survival (mortality selection), mating success (sexual selection), or family size (fecundity selection) of **individuals**. But what of the worker honeybee who gives up her life when danger threatens her hive? Or the mother bird who, feigning injury, flutters away from her nestful of young, thus risking death at the hands of a predator? How can evolution produce genes for such instinctive patterns of behavior when the owner of these genes risk failing the first test of fitness: survival?

A possible solution to this dilemma lies in the effect of such seemingly altruistic behavior on the overall ("inclusive") fitness of the family of the altruistic individual. Linked together be a similar genetic endowment, the altruistic member of the family enhances the chance that many of its own genes will be passed on to future generations by sacrificing itself for the welfare of its relatives. It is interesting to note that most altruistic behavior is observed where the individuals are linked by fairly close family ties. Natural selection working at the level of the family rather than the individual is called **kin selection**.

How good is the evidence for kin selection? Does the behavior of the mother bird really increase her chances of being killed? After all, it may be advantageous to take the initiative in an encounter with a predator that wanders near. But even if she does increase her risk, is this anything more than another example of maternal behavior? Her children are, indeed, her kin. But isn't natural selection simply operating in one of the ways Darwin described: to produce larger mature families?

Perhaps clearer examples of altruism and kin selection are to be found in those species of birds that employ "helpers". One example: Florida scrub jays (*Aphelocoma coerulescens coerulescens*). These birds occupy well-defined territories. When they reach maturity, many of the young birds remain for a time (one to four years) in the territory and help their parents with the raising of additional broods. How self-sacrificing! Should not natural selection have produced a genotype that leads its owners to seek mates and start raising their own families (to receive those genes)?

But the idea of kin selection suggests that the genes guiding their seemingly altruistic behavior have been selected for because they are more likely to be passed on to subsequent generations in the bodies of an increased number of younger brothers and sisters than in the bodies of their own children. To demonstrate that this is so, it is necessary to show that

1. the "helping" behavior of these unmated birds is really a help and that
2. they have truly sacrificed opportunities to be successful parents themselves.

Thanks to the patient observations of Glen Woolfenden, the first point is established. He has shown that parents with helpers raise larger broods than those without. But the second point remains unresolved. Perhaps by waiting until they have gained experience with guarding nests and feeding young and until a suitable territory becomes available, these seemingly altruistic helpers are actually improving their chances of *eventually* raising larger families than they would have if they started right at it. If so, then once again we are simply seeing natural selection working through one of Darwin's criteria of individual fitness: ability to produce larger mature families.

The evolutionary advantage of helping ceases if the young are not actually siblings of the helper. It is well-established (e.g., by DNA analysis) that the females of many species of birds have "extramarital" affairs; that is, produce broods where the young have been sired by more than one male. Interestingly, it turns out that the more promiscuous the females of a given species, the less likely it is that they are assisted by helpers. Conversely, those species that employ helpers tend to be monogamous. (There are a few exceptions.)

Kin Selection in Social Insects

The honeybee and other social insects provide the clearest example of kin selection. They are also particularly interesting examples because of the peculiar genetic relationships among the family members.

Male honeybees (drones) develop from the queen's unfertilized eggs and are haploid. Thus all their sperm will contain exactly the same set of genes. This means that all their daughters will share exactly the same set of **paternal** genes, although they will share — on average — only one-half of their mother's genes. (Human sisters, in contrast, share one-half of their father's as well as one-half of their mother's genes.) So any behavior that favors honeybee sisters (75% of genes shared) will be more favorable to

their genotype than behavior that favors their children (50% of genes shared).

Since that is the case, why bother with children at all? Why not have most of the sisters be sterile workers, caring for their mother as she produces more and more younger sisters, a few of whom will someday be queens? As for their brothers, worker bees share only 25% of their genes with them. Is it surprising, then, that as autumn draws near, the workers lose patience with the lazy demanding ways of their brothers and finally drive them from the hive?

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18.9: The Origin of Life

To account for the origin of life on our earth requires solving several problems:

- How the organic molecules that define life, e.g. amino acids, nucleotides, were created.
- How these were assembled into macromolecules, e.g. proteins and nucleic acids, - a process requiring catalysts.
- How these were able to reproduce themselves.
- How these were assembled into a system delimited from its surroundings (i.e., a cell).

A number of theories address each of these problems. As for the first problem, four scenarios have been proposed. Organic molecules:

1. were synthesized from inorganic compounds in the atmosphere,
2. rained down on earth from outer space,
3. were synthesized at hydrothermal vents on the ocean floor,
4. were synthesized when comets or asteroids struck the early earth.

Scenario 1: Miller's Experiment

Stanley Miller, a graduate student in biochemistry, built the apparatus shown in Figure 18.9.1. He filled it with water (H_2O), methane (CH_4), ammonia (NH_3) and hydrogen (H_2), but no oxygen. He hypothesized that this mixture resembled the atmosphere of the early earth. The mixture was kept circulating by continuously boiling and then condensing the water. The gases passed through a chamber containing two electrodes with a spark passing between them.

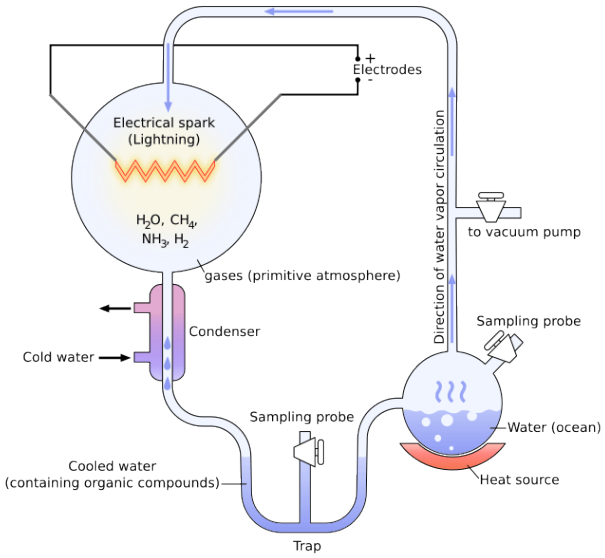


Figure 18.9.1: Miller experiment. (CC BY-SA; Yassine Mrabet).

At the end of a week, Miller used paper chromatography to show that the flask now contained several amino acids as well as some other organic molecules. However, it is now thought that the atmosphere of the early earth was **not** rich in methane and ammonia - essential ingredients in Miller's experiments. In the years since Miller's work, many variants of his procedure have been tried. Virtually all the small molecules that are associated with life have been formed:

- 17 of the 20 amino acids used in protein synthesis, and all the purines and pyrimidines used in nucleic acid synthesis.
- But abiotic synthesis of **ribose -** and thus of **nucleotides -** has been much more difficult. However, success in synthesizing pyrimidine ribonucleotides under conditions that might have existed in the early earth was reported in the 14 May 2009 issue of **Nature**.
- And in 2015, chemists in Cambridge England led by John Sutherland reported that they had been able to synthesize

precursors of 12 of the 20 amino acids and two (of the four) ribonucleotides used by life as well as glycerol-1-phosphate, a precursor of lipids. They created all of these molecules using only hydrogen cyanide (HCN) and hydrogen sulfide (H_2S) irradiated with ultraviolet light in the presence of mineral catalysts.

Scenario 2: Molecules from Outer Space

Astronomers, using infrared spectroscopy, have identified a variety of organic molecules in interstellar space, including methane (CH_4), methanol (CH_3OH), formaldehyde (HCHO), cyanoacetylene (HC_3N) (which in spark-discharge experiments is a precursor to the pyrimidine **cytosine**), polycyclic aromatic hydrocarbonsas well as such inorganic building blocks as carbon dioxide (CO_2), carbon monoxide (CO), ammonia (NH_3), hydrogen sulfide (H_2S), and hydrogen cyanide (HCN).

There have been several reports of producing amino acids and other organic molecules in laboratories by taking a mixture of molecules known to be present in interstellar space such as ammonia (NH_3), carbon monoxide (CO), methanol (CH_3OH) and water (H_2O), hydrogen cyanide (HCN) and exposing it to a temperature close to that of space (near absolute zero) and intense ultraviolet (UV) radiation. Whether or not the molecules that formed terrestrial life arrived here from space, there is little doubt that **organic matter** continuously rains down on the earth (estimated at 30 tons per day).

Alternatively, organic molecules can be transport to Earth via meteorites as demonstrated with the Murchison Meteorite that that fell near Murchison, Australia on 28 September 1969. This meteorite turned out to contain a variety of organic molecules including: purines and pyrimidines, **polyols -** compounds with hydroxyl groups on a backbone of 3 to 6 carbons such as glycerol and glyceric acid (sugars are polyols) and the amino acids listed in Table 18.9.1. The amino acids and their relative proportions were quite similar to the products formed in Miller's experiments.



Murchison meteorite at the The National Museum of Natural History (Washington). (CC SA-BY 3.0; :Basilicofresco).

Table 18.9.1: Representative amino acids found in the Murchison meteorite. Six of the amino acids (blue) are found in all living things, but the others (yellow) are not normally found in living matter here on earth. The same amino acids are produced in discharge experiments like Miller's.

Glycine	Glutamic acid
Alanine	Isovaline
Valine	Norvaline
Proline	N-methylalanine

Aspartic acid	N-ethylglycine

Contamination?

The question is if these molecules identified in the Murchison meteorite were simply terrestrial contaminants that got into the meteorite after it fell to earth? Probably not:

- Some of the samples were collected on the same day it fell and subsequently handled with great care to avoid contamination.
- The polyols contained the isotopes carbon-13 and hydrogen-2 (deuterium) in greater amounts than found here on earth.
- The samples lacked certain amino acids that are found in all earthly proteins.
- Only **L** amino acids occur in earthly proteins, but the amino acids in the meteorite contain both **D** and **L** forms (although **L** forms were slightly more prevalent).

Scenario 3: Deep-Sea Hydrothermal Vents

Some deep-sea hydrothermal vents discharge copious amounts of hydrogen, hydrogen sulfide, and carbon dioxide at temperatures around 100°C. (These are not "black smokers".) These gases bubble up through chambers rich in iron sulfides (FeS, FeS_2). These can catalyze the formation of simple organic molecules like acetate. (And life today depends on enzymes that have Fe and S atoms in their active sites.)

Scenario 4: Laboratory Synthesis of Nucleobases Under Conditions Mimicking the Impact of Asteroids or Comets on the Early Earth

Researchers in the Czech Republic reported in 2014 that they had succeeded in the abiotic synthesis of adenine (A), guanine (G), cytosine (C), and uracil (U) — the four bases found in RNA (an RNA beginning?) and three of the four found in DNA. They achieved this by bombarding a mixture of formamide and clay with powerful laser pulses that mimicked the temperature and pressure expected when a large meteorite strikes the earth. Formamide is a simple substance, CH_3NO, thought to have been abundant on the early earth and containing the four elements fundamental to all life.

Assembling Polymers

Another problem is how **polymers -** the basis of life itself - could be assembled.

- In solution, hydrolysis of a growing polymer would soon limit the size it could reach.
- Abiotic synthesis produces a mixture of L and D enantiomers. Each inhibits the polymerization of the other. (So, for example, the presence of **D** amino acids inhibits the polymerization of **L** amino acids (the ones that make up proteins here on earth).

This has led to a theory that early polymers were assembled on solid, mineral surfaces that protected them from degradation, and in the laboratory polypeptides and polynucleotides (RNA molecules) containing about ~50 units have been synthesized on mineral (e.g., clay) surfaces.

An RNA Beginning?

All metabolism depends on enzymes and, until recently, every enzyme has turned out to be a protein. But proteins are synthesized from information encoded in DNA and translated into mRNA. So here is a chicken-and-egg dilemma. The synthesis of DNA and RNA requires proteins. So proteins cannot be made without nucleic acids and nucleic acids cannot be made without proteins. The discovery that certain RNA molecules have enzymatic activity provides a possible solution. These RNA molecules — called ribozymes — incorporate both the features required of life: storage of information and the ability to act as catalysts.

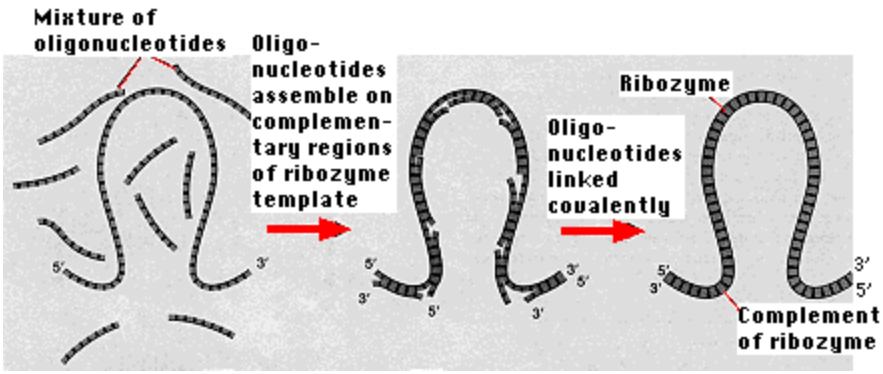


Figure 18.9.2 RNA. (The figure is based on the work of Green and Szostak, Science 258:1910, 1992.)

While no ribozyme in nature has yet been found that can replicate itself, ribozymes have been synthesized in the laboratory that can catalyze the assembly of short oligonucleotides into exact complements of themselves. The ribozyme serves as both the *template* on which short lengths of RNA ("oligonucleotides" are assembled following the rules of base pairing and the *catalyst* for covalently linking these oligonucleotides.

In principal, the minimal functions of life might have begun with RNA and only later did proteins take over the catalytic machinery of metabolism and DNA take over as the repository of the genetic code. Several other bits of evidence support this notion of an original "RNA world":

- Many of the cofactors that play so many roles in life are based on ribose; for example:
  - ATP
  - NAD
  - FAD
  - coenzyme A
  - cyclic AMP
  - GTP
- In the cell, all deoxyribonucleotides are synthesized from ribonucleotide precursors.
- Many bacteria control the transcription and/or translation of certain genes with RNA molecules, not protein molecules.

Reproduction?

Perhaps the earliest form of reproduction was a simple fission of the growing aggregate into two parts - each with identical metabolic and genetic systems intact.

The First Cell?

To function, the machinery of life must be separated from its surroundings - some form of extracellular fluid (ECF). This function is provided by the plasma membrane. Today's plasma membranes are made of a double layer of phospholipids. They are only permeable to small, uncharged molecules like H_2O, CO_2, and O_2. Specialized transmembrane transporters are needed for ions, hydrophilic, and charged organic molecules (e.g., amino acids and nucleotides) to pass into and out of the cell.

However, the same Szostak lab that produced the finding described above reported in the 3 July 2008 issue of **Nature** that fatty acids, fatty alcohols, and monoglycerides - all molecules that can be synthesized under prebiotic conditions - can also form lipid bilayers and these can spontaneously assemble into enclosed vesicles.

Unlike phospholipid vesicles, these

- admit from the external medium charged molecules like nucleotides
- admit from the external medium hydrophilic molecules like ribose
- grow by self-assembly
- are impermeable to, and thus retain, polymers like oligonucleotides.

These workers loaded their synthetic vesicles with a short single strand of deoxycytidine (dC) structured to provide a template for its replication. When the vesicles were placed in a medium containing (chemically modified) dG, these nucleotides entered the vesicles and assembled into a strand of Gs complementary to the template strand of Cs. Here, then, is a simple system that is a plausible model for the creation of the first cells from the primeval "soup" of organic molecules.

From Unicellular to Multicellular Organisms

This transition is probably the easiest to understand.

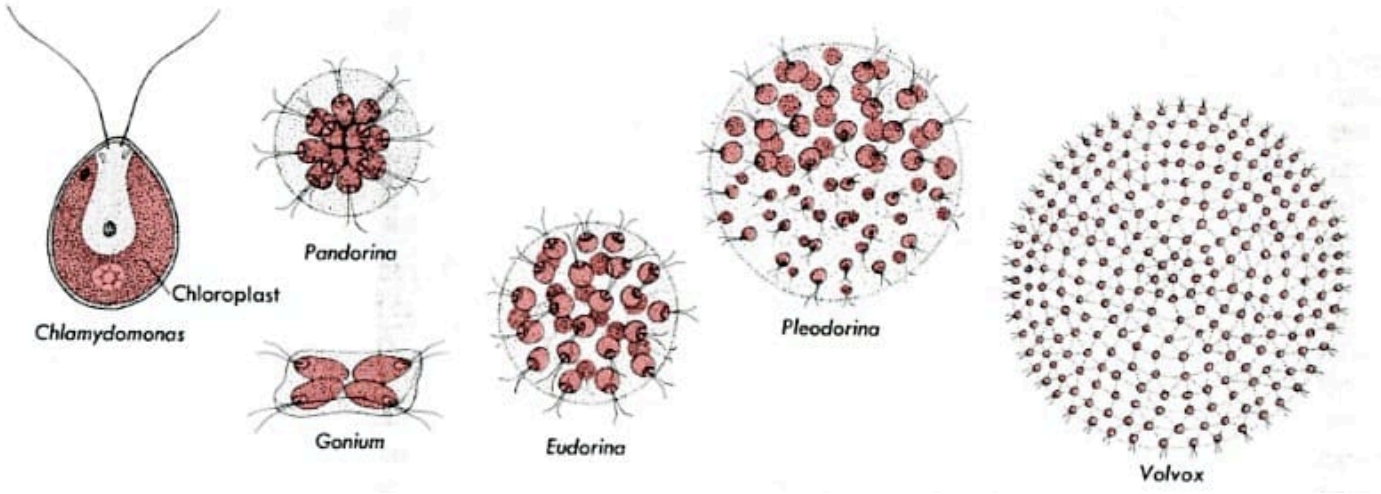


Figure 18.9.3 Unicellular to multicellular organisms

Several colonial flagellated green algae provide a clue. These species are called colonial because they are made up simply of clusters of independent cells. If a single cell of **Gonium**, **Pandorina**, or **Eudorina** is isolated from the rest of the colony, it will swim away looking quite like a **Chlamydomonas** cell. Then, as it undergoes mitosis, it will form a new colony with the characteristic number of cells in that colony.

(The figures are not drawn to scale. Their sizes range from Chlamydomonas which is about 10 µm in diameter - little larger than a human red blood cell - to Volvox whose sphere is some 350 µm in diameter - visible to the naked eye.)

The situation in **Pleodorina** and **Volvox** is different. In these organisms, some of the cells of the colony (most in Volvox) are not able to live independently. If a nonreproductive cell is isolated from a Volvox colony, it will fail to reproduce itself by mitosis and eventually will die. What has happened? In some way, as yet unclear, Volvox has crossed the line separating simple colonial organisms from truly multicellular ones. Unlike Gonium, Volvox cannot be considered simply a colony of individual cells. It is a single **organism** whose cells have lost their ability to live independently. If a sufficient number of them become damaged, the entire sphere of cells will die.

What has Volvox gained? In giving up their independence, the cells of Volvox have become specialists. No longer does every cell carry out all of life's functions (as in colonial forms); instead certain cells specialize to carry out certain functions while leaving other functions to other specialists. In Volvox this process goes no further than having certain cells specialize for reproduction while others, unable to reproduce themselves, fulfill the needs for photosynthesis and locomotion.

In more complex multicellular organisms, the degree of specialization is carried much further. Each cell has one or two precise functions to carry out. It depends on other cells to carry out all the other functions needed to maintain the life of the organism and thus its own.

The specialization and division of labor among cells is the outcome of their history of differentiation. One of the great problems in biology is how differentiation arises among cells, all of which having arisen by mitosis, share the same genes.

The genomes of both Chlamydomonas and Volvox have been sequenced. Although one is unicellular, the other multicellular, they have not only about the same number of protein-encoding genes (14,516 in Chlamydomonas, 14,520 in Volvox) but most of these

are homologous. Volvox has only 58 genes that have no relatives in Chlamydomonas and even fewer unique mRNAs.

At one time, many of us would have expected that a multicellular organism like Volvox with its differentiated cells and complex life cycle would have had many more genes than a single-celled organism like Chlamydomonas. But that turns out not to be the case.

How to explain this apparent paradox? My guess is that just as we have seen in the evolution of animals, we are seeing here that the evolution of organismic complexity is not so much a matter of the evolution of **new genes** but rather the evolution of changes in the control elements (promoters and enhancers) that dictate how and where the basic tool kit of eukaryotic genes will be expressed .

The evidence is compelling that all these organisms are close relatives; that is, belong to the same clade. They illustrate how colonial forms could arise from unicellular ones and multicellular forms from colonial ones.

The Last Universal Common Ancestor (LUCA)?



The 3 kingdoms of contemporary life — archaea, bacteria, and eukaryotes — all share many similarities of their metabolic and genetic systems . Presumably these were present in an organism that was ancestral to these groups: the "LUCA". Although there are not enough data at present to describe LUCA, comparative genomics and proteomics reveal a closer relationship between archaea and eukaryotes than either shares with the bacteria. Except, of course, for the mitochondria and chloroplasts that eukaryotes gained from bacterial endosymbionts. Whether the endosymbionts were acquired before or after a lineage of archaea had acquired a nucleus - and thus started the lineage of eukaryotes - is still uncertain.

Creating Life?

When I headed off to college (in 1949), I wrote an essay speculating on the possibility that some day we would be able to create a living organism from nonliving ingredients. By the time I finished my formal studies in biology — having learned of the incredible complexity of even the simplest organism — I concluded that such a feat could never be accomplished.

Now I'm not so sure.

Several recent advances suggest that we may be getting close to creating life. (But note that these examples represent laboratory manipulations that do not necessarily reflect what may have happened when life first appeared.)

Examples:

- The ability to created membrane-enclosed vesicles that can take in small molecules and assemble them into polymers which remain within the "cell".
- The ability to assemble functional ribosomes — the structures that convert the information encoded in the genome into the proteins that run life — from their components.
- In 2008, scientists at the J. Craig Venter Institute (JCVI) reported (in **Science** 29 February 2008) that they had succeeded in synthesizing a complete bacterial chromosome — containing 582,970 base pairs — starting from single deoxynucleotides. The entire sequence of the genome of *Mycoplasma genitalium* was already known. Using this information, they synthesized some 10,000 short oligonucleotides (each about 50 bp long) representing the entire genitalium genome and then - step by step - assembled these into longer and longer fragments until finally they had made the entire circular DNA molecule that is the genome.

  Could this be placed in the cytoplasm of a living cell and run it?

  The same team showed in the previous year (see **Science** 3 August 2007) that they could insert an entire chromosome from one species of mycoplasma into the cytoplasm of a related species and, in due course, the recipient lost its own chromosome (perhaps destroyed by restriction enzymes encoded by the donor chromosome) and began expressing the phenotype of the donor. In short, they had changed one species into another. But the donor chromosome was made by the donor bacterium, not synthesized in the laboratory. However, there should be no serious obstacle to achieving the same genome transplantation with a chemically-synthesized chromosome.

They've done it! The same team reported on 20 May 2010 in the online *Science* Express that they had successfully transplanted a completely synthetic genome — based on that of *Mycoplasma mycoides* — into the related species *Mycoplasma capricolum*. The recipient strain grew well and soon acquired the phenotype of the *M. mycoides* donor.

- In the 4 April 2014 issue of *Science* (Annaluru, N. *et al.*), a large group of researchers - including many undergraduates at Johns Hopkins University - reported that they had successfully replaced the natural chromosome 3 in *Saccharomyces cerevisiae* (which has 16 chromosomes) with a totally-synthetic chromosome.

  Their procedure:

  1. Chemically synthesize 69- to 79-nt oligonucleotides representing all the stretches of the known chromosome 9 sequence (which contains 316,617 base pairs) **except** for certain sequences such as transposons, many introns, and transfer RNA genes. In addition new, non-native, sequences such as loxP sites were included to aid future manipulations of the genome.
  2. Stitch these together into blocks of ~750 base pairs. This step was done *in vitro* by undergraduates enrolled in the "Build A Genome" class at Johns Hopkins.
  3. Introduce these into yeast cells which ligated them into stretches of DNA containing 2–4 thousand base pairs.
  4. Introduce these stepwise into yeast cells so that they replace the equivalent portions of the native chromosome.
  5. The result: a strain of yeast that grows just as well with its new artificial chromosome (now containing only 272,871 base pairs) as it did before.

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18.10: Mars

In 1975, NASA launched two unmanned landers towards the planet Mars:

- Viking 1 settled gently on Mars on July 20, 1976
- Viking 2 landed on the opposite side of the planet on September 3, 1976.

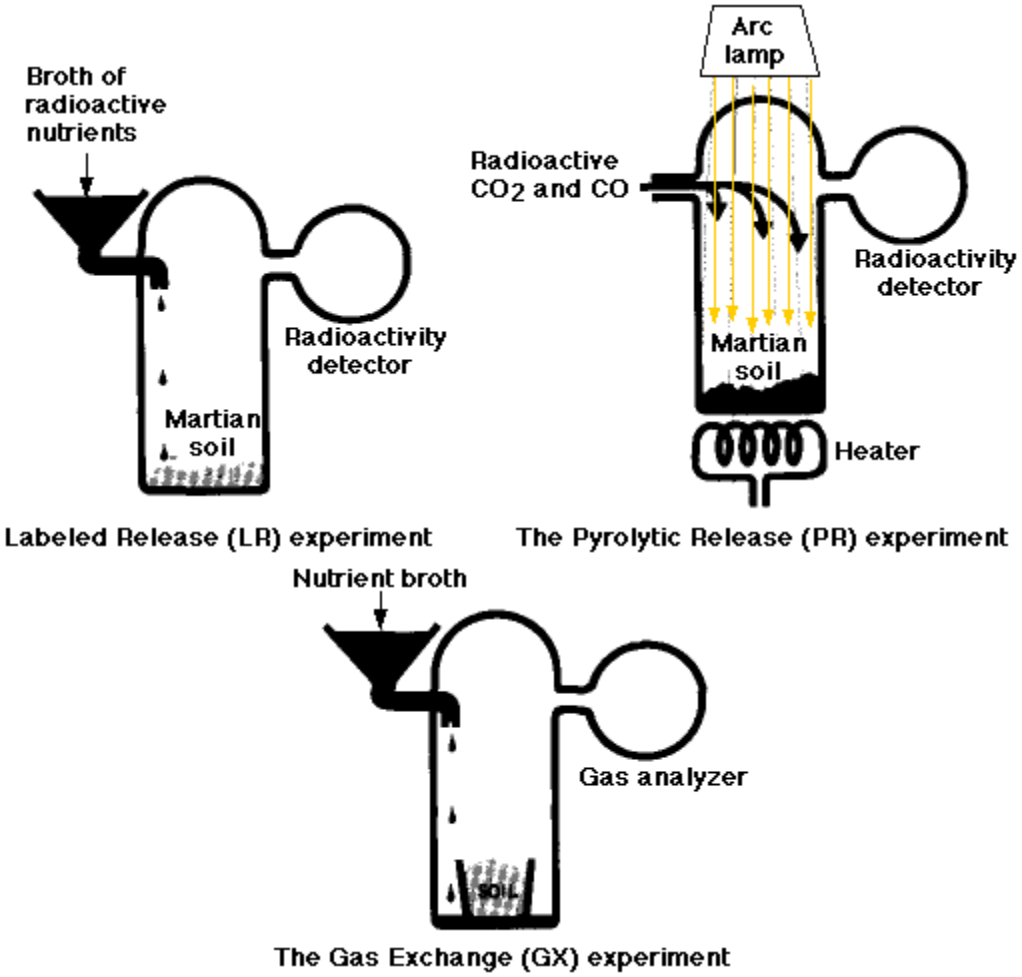
Both Viking missions carried equipment designed to look for evidence of life. There were five different types of instruments:

Television cameras.

No images suggesting the presence of life were ever seen.

A gas chromatograph combined with a mass spectrometer

This apparatus examined the martian soil for the presence of organic molecules. Even though sensitive to concentrations in the parts per billion (ppb) range, no organic matter was detected (except for traces of the solvents that had been used on earth to clean the equipment). Even if organic molecules could be formed on Mars, the intensity of the ultraviolet light at the surface would soon destroy them.



Labeled Release (LR) experiment

The Pyrolytic Release (PR) experiment

The Gas Exchange (GX) experiment

The Labeled-Release (LR) Experiment

Metabolism is a universal property of life on earth. The LR experiment was designed to look for evidence of catabolism by any microorganisms that might have been present in the Martian soil. In this experiment, a soil sample was incubated with a dilute soup of organic molecules (such as the amino acid glycine) which had been synthesized with the radioactive isotope ^{14}C. Over a period of 10 days, the atmosphere above the sample was monitored for the appearance of radioactive gases such as carbon dioxide (CO_2). The results:

- a burst of gas production when the medium was first added
- but not when the soil had been preheated to kill off any microorganisms it might have contained.
- However, gas production did not increase as time went on (as would be expected if living organisms were growing in the medium) and
- later additions produced no additional gas.

Thus most scientists concluded that the gas was produced by nonliving chemistry (brought about by oxidizing agents in the soil). This conclusion was strengthened by similar results using soils from a dry desert in northern Chile. (See Navarro-Gonzalez, R., *et al*, Science, 7 November 2003)

The Pyrolytic-Release (PR) Experiment

The PR experiment was designed to look for evidence of anabolism.; specifically whether there were any microorganisms in the martian soil that could synthesize complex organic molecules from carbon dioxide (CO_2) and carbon monoxide (CO). In this experiment, a mixture of radioactive CO_2 and CO was introduced into a vessel containing a soil sample.
Because anabolism requires energy and the most important source of that energy here on earth is sunlight (for photosynthesis), the incubation mixture was illuminated with a bright arc lamp.
After 5 days,

- any unreacted CO_2 and CO was flushed out of the system and then
- the soil sample was heated to drive off any radioactive organic molecules that might have been synthesized.

The result: organic matter was detected in 7 of 9 runs. However, some positive results were achieved even on runs where the soil had first been heated to such a high temperature that any microorganisms present would have been killed (at least here on earth).

The Gas-Exchange (GEX) Experiment

In this experiment, a known mixture of gases was placed in the chamber along with the soil sample and then analyzed periodically to see if any gases (e.g. CO_2) had disappeared from - or been added to - the mixture.

- In the first part of the experiment, nutrient broth was added to the chamber but not to the soil. There was a rapid release of
  - large amounts of O_2 (which would not be expected from heterotrophic breakdown of organic substrates). This soon subsided.
  - smaller amounts of CO_2 (an expected product of catabolism).
- One week later, more nutrient broth was added; this time directly to the soil. There was another, smaller, release of CO_2 but no release of O_2. The conclusion: the gases were formed by nonbiological chemistry (oxidizing agents again).

So what can we conclude from these data?

The LR, PR, and GEX experiments all produced some positive results.

However:

- All of these involved puzzling ambiguities, failing to behave as similar tests done on earthly soil samples would have.
- All were later shown to be reproducible here on earth by nonbiological chemistry.

So the Viking studies probably did not reveal the presence of life on Mars. But this is not the same as saying that life does not now nor ever did exist on Mars!

Perhaps:

- The upper layers of soil are inhospitable to life.
- Other places on Mars need to be sampled.

The Curiosity Rover

On 6 August 2012, NASA's Curiosity rover landed on Mars. So far, its sampling has revealed evidence of short chains of aliphatic hydrocarbons, chlorobenzene, an aromatic hydrocarbon and nitrates.

The Evidence from Martian Meteorites

Some meteorites are thought — because of their peculiar chemistry - to have reached earth from Mars. One of these ALH84001 (found in the Allan Hills of Antarctica in 1984) has been subjected to intensive analysis for ingredients suggestive of life processes.
In it have been found:

1. polycyclic aromatic hydrocarbons (PAHs). But in most of the Martian meteorites that have been examined, these and other organic molecules have been trapped inside where no living thing could have deposited them, and PAHs and other organic molecules are also found in meteorites arriving from elsewhere in the solar system.
2. minerals within the meteorite (e.g. carbonates, magnetite) that are formed by living organisms here on earth and appear to have been deposited in the rock of the meteorite at some later time in its history;
3. objects that under the scanning electron microscope look like fossils of tiny microorganisms. However, even the largest of these "nanofossils" have diameters of only 100 nanometers (nm) (0.1 µm, about the size of a ribosome). This is smaller than the smallest microorganisms here on earth (the mycoplasmas, with diameters of about 300 nm) and is smaller than the estimates of the minimum diameter (200 nm) needed to provide the volume necessary to build a living cell.

ALH84001 is thought to have landed in Antarctica some 13,000 years ago. But in July 2011, another Martian meteorite landed in the Moroccan desert. With much less time for terrestrial contamination to occur, it may help settle some of the controversy over the significance of the features found in ALH84001.

What would answer our question?

Organic matter is, despite its name, not the exclusive product of life. Many other meteorites contain organic matter and organic molecules can, of course, be synthesized in the laboratory from inorganic precursors. What does distinguish the organic molecules produced by life is the restriction to one enantiomer or the other. For example, all proteins synthesized by living things here on earth use L-amino acids exclusively. Synthesis of amino acids in the chemistry laboratory produces a 50:50 mixture (called a racemic mixture) of the L- and the D- forms. There is nothing to suggest that life couldn't work just as well with D-amino acids. What is unlikely is the ability of proteins (e.g. enzymes) to be able to function if they are made from a mixture of L- and D-enantiomers. So if martian soil should reveal the presence of all-L (or all-D) enantiomers, this would be powerful evidence that life had produced them.

However,

- Whether tested on Mars itself, or on samples returned to earth, rigorous care must be taken to ensure that there is no contamination by terrestrial molecules.
- There is some evidence that even nonbiological synthesis in space may favor one enantiomer over the other. Four amino acids in the Murchison meteorite, which no one suggests have a biological origin, show a 7–9% excess of the L-form over the D-form.
- Over time, even in the cold, dry climate of Mars, a population of L- (or D-) enantiomers will spontaneously break down into a racemic (50:50) mixture and thus obscure a biological origin.

The case for life on Mars - whether today or in the past - is neither proven nor disproven.

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18.11: Endosymbiosis

The endosymbiosis theory postulates that the **mitochondria** of eukaryotes evolved from an aerobic bacterium (probably related to the rickettsias) living within an archaeal host cell and the **chloroplasts** of red algae, green algae, and plants evolved from an endosymbiotic cyanobacterium living within a mitochondria-containing eukaryotic host cell.

The Evidence

- Both mitochondria and chloroplasts can arise only from preexisting mitochondria and chloroplasts. They cannot be formed in a cell that lacks them because nuclear genes encode only some of the proteins of which they are made.
- Both mitochondria and chloroplasts have their own genome, and it resembles that of bacteria not that of the nuclear genome.
  - Both genomes consist of a **single circular molecule of DNA**.
  - There are **no histones** associated with the DNA.
- Both mitochondria and chloroplasts have their own protein-synthesizing machinery, and it more closely resembles that of bacteria than that found in the cytoplasm of eukaryotes.
  - The first amino acid of their transcripts is always **fMet** as it is in bacteria (not methionine [Met] that is the first amino acid in eukaryotic proteins).
  - A number of antibiotics (e.g., **streptomycin**) that act by blocking protein synthesis in bacteria also block protein synthesis within mitochondria and chloroplasts. They do not interfere with protein synthesis in the cytoplasm of the eukaryotes.
  - Conversely, inhibitors (e.g., **diphtheria toxin**) of protein synthesis by eukaryotic ribosomes do not - sensibly enough - have any effect on bacterial protein synthesis nor on protein synthesis within mitochondria and chloroplasts.
  - The antibiotic **rifampicin**, which inhibits the RNA polymerase of bacteria, also inhibits the RNA polymerase within mitochondria. It has no such effect on the RNA polymerase within the eukaryotic nucleus.

The Mitochondrial Genome

The genome of human mitochondria contains 16,569 base pairs of DNA organized in a closed circle (Figure 18.11.1). These encode 2 ribosomal RNA (rRNA), molecules, 22 transfer RNA (tRNA) molecules, and 13 polypeptides. The 13 polypeptides participate in building several protein complexes embedded in the inner mitochondrial membrane and include 7 subunits that make up the mitochondrial NADH dehydrogenase, 3 subunits of cytochrome c oxidase, 2 subunits of ATP synthase, and cytochrome b.

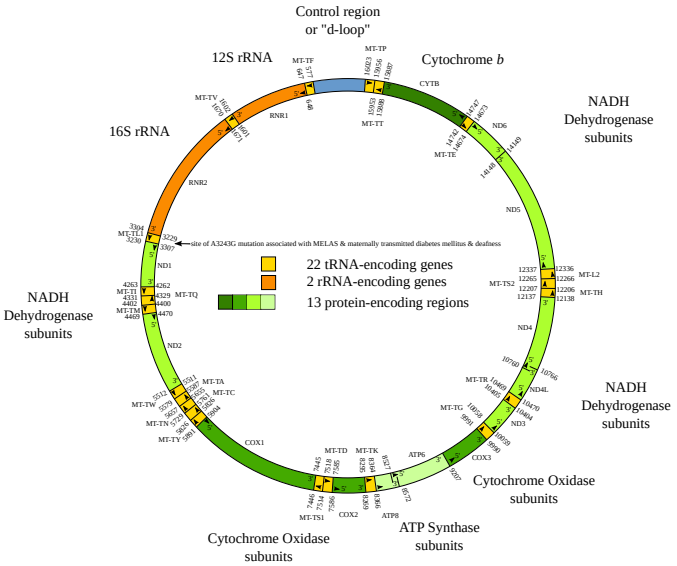


Figure 18.11.1: Human mitochondrial genome, a 16,569-bp (base pair) circle of double-stranded DNA containing 37 genes, specifing 2 ribosomal RNAs , 22 transfer RNAs and 13 polypeptides. Public Domain.

All these gene products are used within the mitochondrion, but the mitochondrion also needs >900 different proteins as well as some mRNAs and tRNAs encoded by nuclear genes. The proteins (e.g., cytochrome c and the DNA polymerases used within the mitochondrion) are synthesized in the cytosol and then imported into the mitochondrion.

The Chloroplast Genome

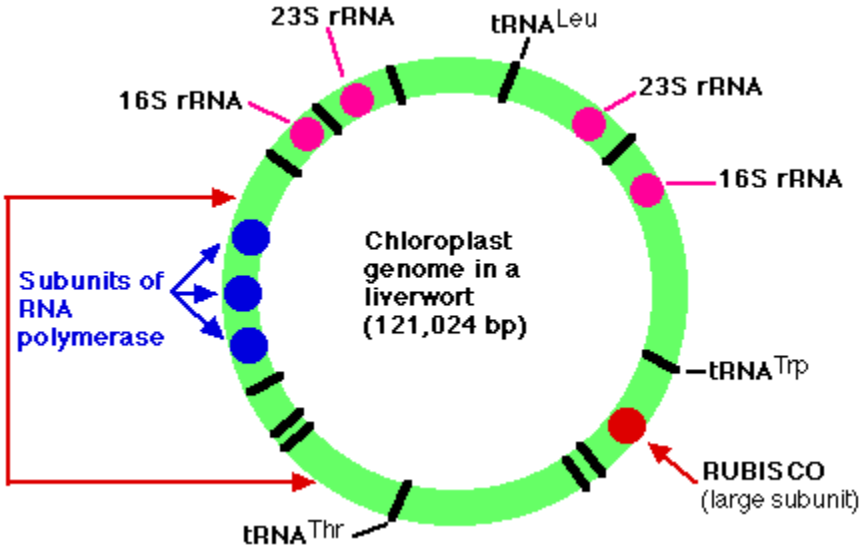


Figure 18.11.2 ChlDNA

The genome of the chloroplasts found in ***Marchantia polymorpha*** (a liverwort, one of the Bryophyta) contains 121,024 base pairs in a closed circle. These make up some 128 genes which include:

- duplicate genes encoding each of the four subunits (23S, 16S, 4.5S, and 5S) of the **ribosomal RNA** (**rRNA**) used by the chloroplast
- 37 genes encoding all the transfer RNA (tRNA) molecules used for translation within the chloroplast. Some of these are represented in the figure by black bars (a few of which are labeled).
- 4 genes encoding some of the subunits of the RNA polymerase used for transcription within the chloroplast (3 of them shown in blue)
- a gene encoding the large subunit of the enzyme **RUBISCO** (ribulose bisphosphate carboxylase oxygenase)
- 9 genes for components of photosystems I and II
- 6 genes encoding parts of the chloroplast ATP synthase
- genes for 19 of the ~60 proteins used to construct the chloroplast ribosome
- All these gene products are used within the chloroplast, but all the chloroplast structures also depend on proteins **RUBISCO**, for example, the enzyme that adds CO_2 to ribulose bisphosphate to start the Calvin cycle, consists of multiple copies of two subunits:
  - Encoded by nuclear genes translated in the cytosol, and imported into the chloroplast.
  - A large one encoded in the chloroplast genome and synthesized within the chloroplast, and a small subunit encoded in the nuclear genome and synthesized by ribosomes in the cytosol. The small subunit must then be imported into the chloroplast.
- The arrangement of genes shown in the figure is found not only in the Bryophytes (mosses and liverworts) but also in the lycopsids (e.g., **Lycopodium** and **Selaginella)**. In all other plants, however, the portion of DNA bracketed by the red arrows on the left is inverted. The same genes are present but in inverted order. The figure is based on the work of Ohyama, K., et al., **Nature**, **322**:572, 7 Aug 1986; and Linda A. Raubeson and R. K. Jansen, **Science**, **255**:1697, 27 March 1992.
- The evolution of the eukaryotic chloroplast by the endosymbiosis of a cyanobacterium in a mitochondria-containing eukaryotic host cell led to the evolution of the green algae and plants as described above, red algae, and glaucophytes; a small group of unicellular algae.

Secondary Endosymbiosis: Eukaryotes Engulfing Eukaryotes

The Nucleomorph

Once both heterotrophic and photosynthetic eukaryotes had evolved, the former repeatedly engulfed the latter to exploit their autotrophic way of life. Many animals living today engulf algae for this purpose. Usually the partners in these mutualistic relationships can be grown separately. However, a growing body of evidence indicates that the **chloroplasts** of some algae have not been derived by engulfing cyanobacteria in a primary endosymbiosis like those discussed above, but by engulfing **photosynthetic eukaryotes** (Figure 18.11.3). This is called **secondary endosymbiosis**. It occurred so long ago that these endosymbionts cannot be cultured away from their host.

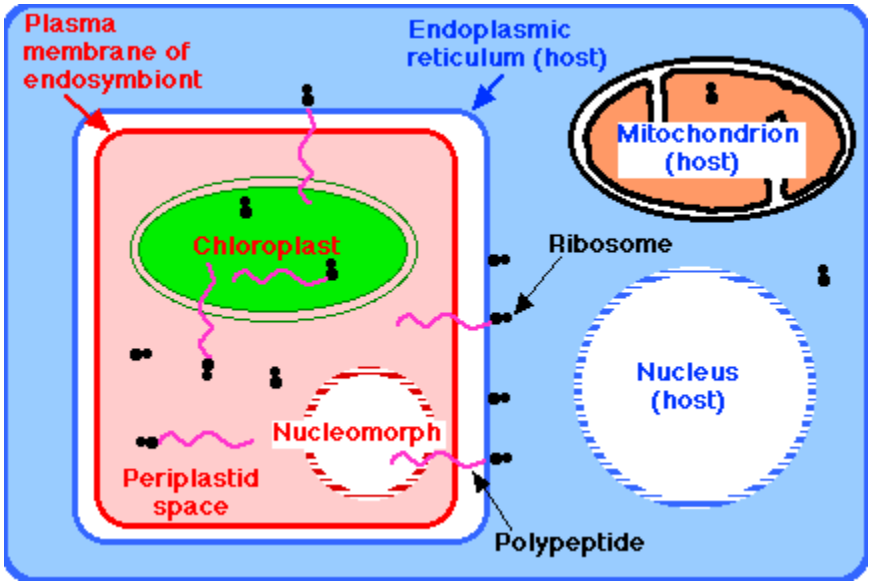


Figure 18.11.3: Secondary endosymbiosis

In two groups, the eukaryotic nature of the endosymbiont can be seen by its retention of a vestige of a nucleus (called its **nucleomorph**). A group of unicellular, motile algae called **cryptomonads** appear to be the evolutionary outcome of a nonphotosynthetic eukaryotic flagellate (i.e., a protozoan) engulfing a **red alga** by endocytosis. Another tiny group of unicellular algae, called **chlorarachniophytes**, appear to be the outcome of a flagellated protozoan having engulfed a **green** alga.

The result in both cases: a motile, autotrophic cell containing its own nucleus, its own mitochondria, and its own endoplasmic reticulum. The latter of which contains the endosymbiont with:

- its own plasma membrane
- its own cytoplasm, the **periplastid space**
- its own ribosomes
- its own chloroplast, and
- its **nucleomorph** - only a vestige of its original nucleus, but still surrounded by a nuclear envelope perforated with nuclear pore complexes and containing a tiny but still-functioning genome.

The Four Genomes of *Guillardia theta*

The cryptomonad Guillardia theta contains four different genomes:

- its own nuclear genome; by far the largest with 87.2×10^6 base pairs (bp) of DNA
- the genome of its mitochondria (48,000 bp)
- the genome of the chloroplast in its endosymbiont (121,000 bp)
- the genome of the nucleomorph (551,264 bp)

Susan Douglas and her colleagues reported (in the 26 April 2001 issue of **Nature**) the completely-sequenced genome of the

nucleomorph.

- It contains 3 small chromosomes with
- 47 genes for nonmessenger RNAs (rRNA, tRNA, snRNA)
- 464 genes for messenger RNA; that is, encoding proteins such as
  - 65 proteins for its own ribosomes
  - 30 for its chloroplast (a small fraction of the hundreds needed)
  - a variety of proteins needed within the nucleomorph, including
    - DNA licensing factors
    - histones
    - proteins needed for DNA replication (but no genes for DNA polymerases, which must be translated by and imported from the host ribosomes)

The genes are crowded closely on the three chromosomes. In fact, 44 of them overlap each other. Only 17 genes contain introns, and these are very small.

Genome Interactions in *Guillardia theta*

Millions of years of evolution have resulted in a complex but precisely-orchestrated array of interactions between the 4 genomes. For example:

- The chloroplast needs proteins synthesized by 3 different genomes: its own, the nucleomorph's, and the host's.
- The nucleomorph genome has given up all (but one) of its genes encoding enzymes for general metabolic functions; the endosymbiont now depends on those encoded by the host nucleus.
- The nucleomorph itself also depends on genes (e.g., for DNA polymerases) residing in the host nucleus.

The Apicoplast

The apicoplast (short for "apicomplexan plastid") is a solitary organelle found in the apicomplexan protists: "sporozoans" like ***Plasmodium falciparum*** (and the other agents of malaria) and *Toxoplasma gondii*.

Features:

- Essential - the organisms cannot survive without it.
- Encased by 4 membranes.
- Contains its own genome, a circular molecule of DNA (35,000 base pairs) which encodes
  - ~ 30 proteins
  - a full set of tRNAs plus some other RNAs
- Only a few functions have been discovered, but these include
  - anabolic metabolism such as the synthesis of fatty acids
  - repair, replication, transcription, and translation of its genes
- Clearly 30 proteins are not enough to accomplish so many functions so the apicoplast has to import from the cytosol ~500 nuclear-encoded proteins.

The apicoplast is the product of an ancient endosymbiosis in which the eukaryotic ancestor engulfed a unicellular alga - probably a red alga - with a solitary chloroplast. Over time, the nucleus was lost (no residual nucleomorph) as well as many features of the chloroplast (including its ability to perform photosynthesis).

Secondary Symbiosis can Still Occur

Two Japanese scientists have discovered a heterotrophic flagellate that engulfs a unicellular green alga that lives freely in the surrounding water. Once inside, the alga loses its flagella and cytoskeleton; the host loses its feeding apparatus. Moreover, the host switches from heterotrophic to autotrophic nutrition (photosynthesis) and the host becomes capable of phototaxis. When the host divides by mitosis, only one daughter cell gets the plastid. The other cell regrows the feeding apparatus and is ready to engulf another alga.

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18.12: Geologic Eras

History of life as revealed by the fossil record

With help from molecular phylogenies:

Eras	Periods	Epochs	Aquatic Life	Terrestrial Life
With approximate starting dates in millions of years ago in parentheses. Geologic features in green				
Cenozoic (66) The "Age of Mammals"	Quaternary (2.6)	Holocene		Humans in the new world
		Pleistocene	Periodic glaciation	First humans
			Continental drift continues	
	Neogene (23)	Pliocene	Atmospheric oxygen reaches today's level (21%)	Hominids
		Miocene		Adaptive radiation of birds, continued radiation of mammals
	Paleogene (66)	Oligocene	All modern groups present	
		Eocene		
		Paleocene		
Mesozoic (251) The "Age of Reptiles"	Cretaceous (146)	Still attached: N. America & N. Europe; Australia & Antarctica; Mass extinction of both aquatic and terrestrial life at the end		
			Modern bony fishes	Extinction of dinosaurs and pterosaurs; first snakes
			Extinction of ammonites, plesiosaurs, ichthyosaurs	Rise of angiosperms
		Africa & S. America begin to drift apart		
	Jurassic (200)		Plesiosaurs, ichthyosaurs abundant; first diatoms	*Archaeopteryx*; dinosaurs dominant but mammals (Eutheria) begin to diversify
			Ammonites again abundant	First lizards
			Skates, rays, and bony fishes abundant	Adaptive radiation of dinosaurs
			Pangaea splits into Laurasia and Gondwana; atmospheric oxygen drops to ~13%	
	Triassic (251)		Mass extinctions at the end.	Mass extinctions at the end. First mammals Adaptive radiation of reptiles: thecodonts, therapsids, turtles, crocodiles, first dinosaurs
			Ammonites abundant at first	
			Rise of bony fishes	

Eras	Periods	Epochs	Aquatic Life	Terrestrial Life		
	Paleozoic (542)	Permian (299)	Periodic glaciation and arid climate; atmospheric oxygen reaches ~30%. Volcanic eruptions killed off 90% of marine species at end.			
			Extinction of trilobites	Reptiles abundant. Cycads, conifers, ginkgos		
Pennsylvanian (320)		Warm, humid climate Together the Pennsylvanian and Mississippian make up the "Carboniferous"; also called the "Age of Amphibians"	Ammonites, bony fishes	First reptiles Coal swamps		
Mississippian (359)			Adaptive radiation of sharks	Forests of lycopsids, sphenopsids, and seed ferns Amphibians abundant Adaptive radiation of the insects (Hexapoda)		
			Atmospheric oxygen begins to rise as organic matter is buried, not respired			
Devonian (416) The "Age of Fishes"		Extensive inland seas	Cartilaginous and bony fishes abundant. Ammonites, nautiloids, ostracoderms, eurypterids	Ferns, lycopsids, and sphenopsids First gymnosperms First amphibians		
				Silurian (443)	Mild climate; inland seas	First bony fishes First myriapods and chelicerates
Ordovician (485)		Mild climate, inland seas	Trilobites abundant	Fungi present First plants (liverworts?) First insects		
Cambrian (541)			First vertebrates (jawless fishes). Eurypterids, crustaceans mollusks, echinoderms, sponges, cnidarians, annelids, and tunicates present. Trilobites dominant.	No fossils of terrestrial eukaryotes, but phylogenetic trees suggest that lichens, mosses, perhaps even vascular plants were present.		
		Periodic glaciation				
Proterozoic (2500)		Ediacaran (635)		Fossil evidence of multicellular algae, fungi, and bilaterian invertebrates		
				Evidence of eukaryotes ~1.8 x10^9 years ago		
Archaean (3600)			Evidence of archaea and bacteria ~3.5 x10^9 years ago			

The Geologic and Evolutionary Record

A remarkable feature of the table above is how often evolutionary changes coincided with geologic changes on the earth. But consider that changes in geology (e.g., mountain formation or lowering of the sea level) cause changes in climate, and together these alter the habitats available for life. Two types of geologic change seem to have had especially dramatic effects on life: **continental drift** and the **impact of asteroids**

Continental Drift

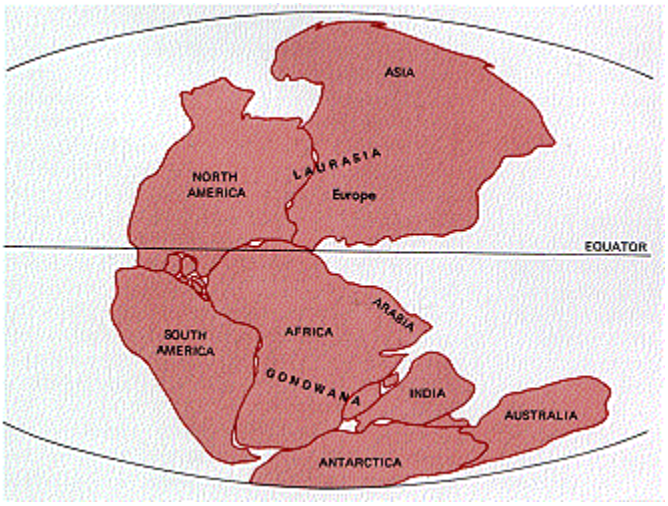


Figure 18.12.1 Pangaea

A body of evidence, both geological and biological, supports the conclusion that 200 million years ago, at the start of the **Mesozoic era**, all the continents were attached to one another in a single land mass, which has been named **Pangaea**. This drawing of Pangaea (adapted from data of R. S. Dietz and J. C. Holden) is based on a computer-generated fit of the continents as they would look if the sea level were lowered by 6000 feet (~1800 meters). During the **Triassic**, Pangaea began to break up, first into two major land masses:

- **Laurasia** in the Northern Hemisphere
- **Gondwana** in the Southern Hemisphere.

The present continents separated at intervals throughout the remainder of the Mesozoic and through the Cenozoic, eventually reaching the positions they have today. Let us examine some of the evidence.

Shape of the Continents

The east coast of South America and the west coast of Africa and are strikingly complementary. This is even more dramatic when one tries to fit the continents together using the boundaries of the continental slopes, e.g., 6000 feet (~1800 meters) down, rather than the shorelines.

Geology

- In both mineral content and age, the rocks in a region on the east coast of Brazil match precisely those found in Ghana on the west coast of Africa.
- The low mountain ranges and rock types in New England and eastern Canada appear to be continued in parts of Great Britain, France, and Scandinavia.
- India and the southern part of Africa both show evidence of periodic glaciation during Paleozoic times (even though both are now close to the equator). The pattern of glacial deposits in the two regions not only match each other but also glacial deposits found in South America, Australia, and Antarctica.

Fossils

- Fossil reptiles found in South Africa are also found in Brazil and Argentina.
- Fossil amphibians and reptiles found in Antarctica are also found in South Africa, India, and China.
- Most of the marsupials alive today are confined to South America and Australia. But if these two continents were connected by Antarctica in the Mesozoic, one might expect to find fossil marsupials there. In March 1982, this prediction was fulfilled with the discovery in Antarctica of the remains of **Polydolops**, a 9-ft (2.7 m) marsupial.

The Impact Hypothesis

The Cretaceous period, the last period of the Mesozoic, marked the end of the Age of Reptiles. It was followed by the Cenozoic era, the Age of Mammals. Although extinctions have occurred throughout the history of life, an extraordinary number of them occurred in a relatively brief period at the end of the Cretaceous. Why?

The Alvarez Theory

Louis Alvarez, his son Walter, and their colleagues proposed that a giant asteroid or comet striking the earth some 66 million years ago caused the massive die-off at the end of the Cretaceous. Presumably, the impact generated so much dust and gases that skies were darkened all over the earth, photosynthesis declined, and worldwide temperatures dropped. The outcome was that as many as 75% of all species — including all dinosaurs — became extinct.

The key piece of evidence for the Alvarez hypothesis was the finding of thin deposits of clay containing the element iridium at the interface between the rocks of the Cretaceous and those of the Paleogene period (called the K-Pg boundary after the German word for Cretaceous). Iridium is a rare element on earth (although often discharged from volcanoes), but occurs in certain meteorites at concentrations thousands of times greater than in the earth's crust.

After languishing for many years, the Alvarez theory gained strong support from the discovery in the 1990s of the remains of a huge (180 km in diameter) crater in the Yucatan Peninsula that dated to 65 million years ago.

The abundance of sulfate-containing rock in the region suggests that the impact generated enormous amounts of sulfur dioxide (SO_2), which later returned to earth as a bath of acid rain. A smaller crater in Iowa, formed at the same time, many have contributed to the devastation. Perhaps during this period the earth passed through a swarm of asteroids or a comet and the repeated impacts made the earth uninhabitable for so many creatures of the Mesozoic.

Other Impacts

A mass extinction of non-dinosaur reptiles occurred earlier, at the end of the **Triassic**. It was followed by a great expansion in the diversity of dinosaurs. The recent discovery of a layer enriched in iridium in rocks formed at the boundary between the Triassic and Jurassic suggests that impact from an asteroid or comet may have been responsible then just as it was at the K-Pg boundary.

The largest extinction of all time occurred still earlier at the end of the Permian period. There is evidence off the coast of Australia that a huge impact there may have contributed to the extinctions at the Permian-Triassic (P-T) boundary.

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UNIT 19: THE DIVERSITY OF LIFE

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19.1.2: Protists

19.1.3: Ciliates

19.1.4: Volvox

19.1.5: Diversity and Evolutionary Relationships of the Plants

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19.1: EUKARYOTIC LIFE



- Topic hierarchy

19.1.1: Taxonomy

19.1.2: Protists

19.1.3: Ciliates

19.1.4: Volvox

19.1.5: Diversity and Evolutionary Relationships of the Plants

19.1.6: Arabidopsis Thaliana - A Model Organism

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19.1.11: Drosophila Melanogaster

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19.1.13: Vertebrates

19.1.14: Zebrafish

19.1.15: Monotremes

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19.1.1: Taxonomy

At least 1.7 million species of living organisms have been discovered, and the list grows longer every year (especially of insects in the tropical rain forest). How are they to be classified? Ideally, classification should be based on **homology**; that is, **shared characteristics that have been inherited from a common ancestor**. The more recently two species have shared a common ancestor, the more homologies they share and the more similar these homologies are. Until recent decades, the study of homologies was limited to anatomical structures and pattern of embryonic development. However, since the birth of molecular biology, homologies can now also be studied at the level of proteins and DNA.

Anatomical homology: an example

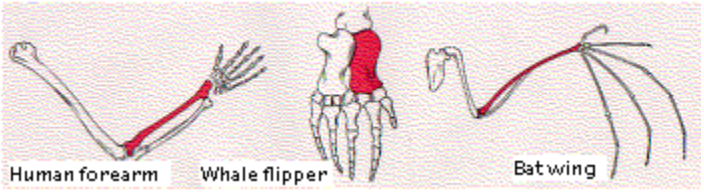


Figure 19.1.1.1 Forelimbs

The figure shows the bones in the forelimbs of three mammals: human, whale, and bat (obviously not drawn to the same scale!). Although used for such different functions as throwing, swimming, and flying, the same basic structural plan is evident in them all. In each case, the bone shown in color is the **radius**. Body parts are considered **homologous** if they have

- the same basic structure
- the same relationship to other body parts
- develop in a similar manner in the embryo

It seems unlikely that a single pattern of bones represents the best possible structure to accomplish the functions to which these forelimbs are put. However, if we interpret the persistence of the basic pattern as evidence of inheritance from a common ancestor, we see that the various modifications are adaptations of the plan to the special needs of the organism. It tells us that **evolution is opportunistic**, working with materials that have been handed down by inheritance.

Protein Sequences

Protein sequencing provides a tool for establishing homologies from which genealogies can be constructed and phylogenetic trees drawn. Here are two examples.

Hemoglobins

An example of molecular homology.

Human beta chain	0
Gorilla	1
Gibbon	2
Rhesus monkey	8
Dog	15
Horse, cow	25
Mouse	27
Gray kangaroo	38
Chicken	45
Frog	67
Lamprey	125
Sea slug (a mollusk)	127
Soybean (leghemoglobin)	124

The numbers represent the number of amino acid differences between the beta chain of human hemoglobin and the hemoglobins of the other species. In general, the number is inversely proportional to the closeness of kinship. All the values listed are for the beta chain except for the last three, in which the distinction between alpha and beta chains does not occur. The human beta chain contains 146 amino acid residues, as do most of the others.

Cytochrome c

Cytochrome c is part of the electron transport chain down which electrons are passed to oxygen during cellular respiration. Cytochrome c is found in the mitochondria of every aerobic eukaryote - animal, plant, and protist. The amino acid sequences of many of these have been determined, and comparing them shows that they are related. Human cytochrome c contains 104 amino acids, and 37 of these have been found at equivalent positions in every cytochrome c that has been sequenced. We assume that each of these molecules has descended from a precursor cytochrome in a primitive microbe that existed over 2 billion years ago. In other words, these molecules are **homologous**.

The first step in comparing cytochrome c sequences is to align them to find the maximum number of positions that have the same amino acid. Sometimes gaps are introduced to maximize the number of identities in the alignment (none was needed in this table). Gaps correct for insertions and deletions that occurred during the evolution of the molecule.

This table shows the N-terminal 22 amino acid residues of human cytochrome c with the corresponding sequences from six other organisms aligned beneath. A dash indicates that the amino acid is the same one found at that position in the human molecule. All the vertebrate cytochromes (the first four) start with glycine (Gly). The ***Drosophila***, wheat, and yeast cytochromes have several amino acids that precede the sequence shown here (indicated by <<<). In every case, the heme group of the cytochrome is attached to Cys-14. and Cys-17 (human numbering). In addition to the two Cys residues, Gly-1, Gly-6, Phe-10, and His-18 are found at the equivalent positions in every cytochrome c that has been sequenced.

We assume that the more identities there are between two molecules, the more recently they have evolved from a common ancestral molecule and thus the closer the kinship of their owners. Thus the cytochrome c of the rhesus monkey is identical to that of humans except for one amino acid, whereas yeast cytochrome c differs from that of humans at 44 positions. (There are no differences between the cytochrome c of humans and that of chimpanzees.)

Molecular homology of cytochrome c (see three-letter code of amino acids)

		1					6				10				14			17	18		20		
Human		Gly	Asp	Val	Glu	Lys	Gly	Lys	Lys	Ile	Phe	Ile	Met	Lys	Cys	Ser	Gln	Cys	His	Thr	Val	Glu	Lys
Pig		-	-	-	-	-	-	-	-	-	-	Val	Gln	-	-	Ala	-	-	-	-	-	-	-
Chicken		-	-	Ile	-	-	-	-	-	-	-	Val	Gln	-	-	-	-	-	-	-	-	-	-
Dogfish		-	-	-	-	-	-	-	-	Val	-	Val	Gln	-	-	Ala	-	-	-	-	-	-	Asn
Drosophila	<<<	-	-	-	-	-	-	-	-	Leu		Val	Gln	Arg		Ala	-	-	-	-	-	-	Ala
Wheat	<<<	-	Asn	Pro	Asp	Ala	-	Ala	-	-	-	Lys	Thr	-	-	Ala	-	-	-	-	-	Asp	Ala
Yeast	<<<	-	Ser	Ala	Lys	-	-	Ala	Thr	Leu	-	Lys	Thr	Arg	-	Glu	Leu	-	-	-	-	-	-

Phylogenetic trees

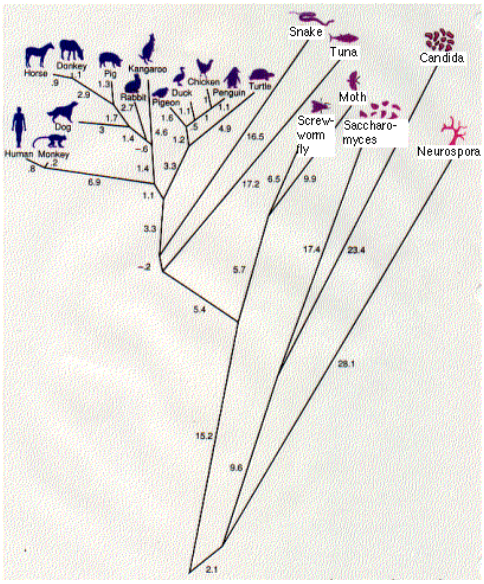


Figure 19.1.1.2 Phylogenetic trees

With such information, one can reconstruct an evolutionary history of the molecule and thus of their respective owners. This requires

- using the genetic code to determine the minimum number of nucleotide substitutions in the DNA of the gene needed to derive one protein from another
- a powerful computer program to search for the shortest paths linking the molecules together

The result is a phylogenetic tree. This one (the work of Walter M. Fitch and Emanuel Margoliash) shows the relationship between 20 species of eukaryotes. The numbers represent the minimum number of nucleotide substitutions in the gene for cytochrome c needed to produce these 20 proteins from a series of hypothetical ancestral genes at the various branching points (nodes).

The tree corresponds quite well to what we have long believed to be the evolutionary relationships among the vertebrates. But there are some anomalies. It indicates, for example, that the primates (humans and monkeys) split off before the split separating the kangaroo, a marsupial, from the other placental mammals. This is certainly wrong. But sequence analysis of other proteins can resolve such discrepancies.

Cytochrome c is an ancient molecule, and it has evolved very slowly. Even after more than 2 billion years, one-third of its amino acids are unchanged. This conservatism is a great help in working out the evolutionary relationships between distantly-related creatures like fish and humans.

But what of humans and the great apes? Their cytochrome c molecules are identical and can tell us nothing about evolutionary relationships. However, some proteins have evolved much more rapidly than cytochrome c, and these can be used to decipher recent evolutionary events. During blood clotting, short peptides are cut from **fibrinogen** converting it into insoluble **fibrin**. Once removed, these **fibrinopeptides** have no further function. They have been pretty much free from the rigors of natural selection and have, consequently, diverged rapidly during evolution. So they provide data useful in sorting out the twigs of phylogenetic trees of mammals, for example.

DNA-DNA Hybridization

As we saw in the comparison of human and kangaroo cytochrome c, a single molecule provides only a narrow window for glimpsing evolutionary relationships.

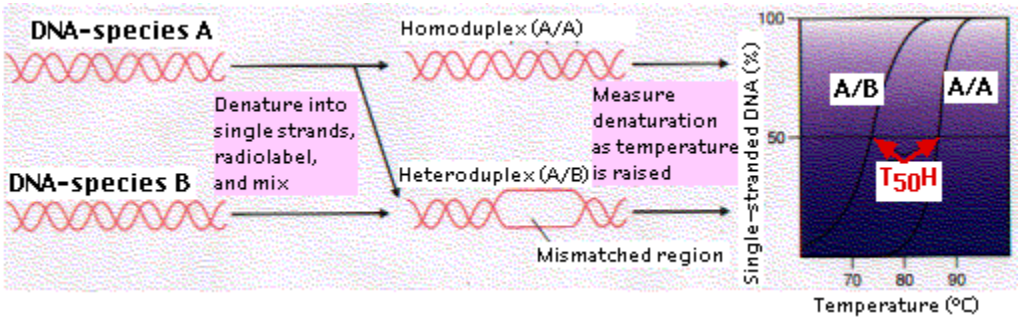


Figure 19.1.1.3 DNA-DNA hybridization

The technique of DNA-DNA hybridization provides a way of comparing the **total genome** of two species. Let us examine the procedure as it might be used to assess the evolutionary relationship of **species B** to **species A**:

- The total DNA is extracted from the cells of each species and purified.
- For each, the DNA is heated so that it becomes denatured into single strands (**ssDNA**).
- The temperature is lowered just enough to allow the multiple short sequences of repetitive DNA to rehybridize back into double-stranded DNA (**dsDNA**).
- The mixture of ssDNA (representing single genes) and dsDNA (representing repetitive DNA) is passed over a column packed with hydroxyapatite. The **dsDNA sticks** to the hydroxyapatite; **ssDNA does not** and flows right through. The purpose of this step is to be able to compare the information-encoding portions of the genome — mostly genes present in a single copy — without having to worry about varying amounts of noninformative repetitive DNA.
- The ssDNA of **species A** is made radioactive.
- The radioactive ssDNA is then allowed to rehybridize with nonradioactive ssDNA of the same species (**A**) as well as — in a separate tube — the ssDNA of species **B**.
- After hybridization is complete, the mixtures (**A/A**) and (**A/B**) are individually heated in small (2°–3°C) increments. At each higher temperature, an aliquot is passed over hydroxyapatite. Any radioactive strands (**A**) that have separated from the DNA duplexes pass through the column, and the amount is measured from their radioactivity.
- A graph showing the percentage of ssDNA at each temperature is drawn.
- The temperature at which 50% of the DNA duplexes (dsDNA) have been denatured ($T_{50}H$) is determined.

As the figure shows, the curve for **A/B** is to the left of **A/A**, i.e., duplexes of **A/B** separated at a lower temperature than those of **A/A**. The sequences of A/A are precisely complementary so all the hydrogen bonds between complementary base pairs (A-T, C-G) must be broken in order to separate the strands. But where the gene sequences in **B** differ from those in **A**, no base pairing will have occurred and denaturation is easier.

Thus DNA-DNA hybridization provides genetic comparisons integrated over the entire genome. Its use has cleared up several puzzling taxonomic relationships. DNA-DNA hybridization can also be used to compare genomes of mixed populations of organisms. For example, when all the bacteria are extracted from 10 g of uncontaminated soil (there are about 10^{10} cells in it!), the DNA extracted and purified from the bacteria and subjected to DNA-DNA hybridization analysis, the resulting curves indicate that there are over a million different species in the soil sample, although the population is dominated by only a few of these.

Chromosome Painting

Another way to compare entire genomes is to attach a fluorescent label to the DNA of individual chromosomes of one species (e.g., human) and expose the chromosomes of another species to it. Regions of gene homology will hybridize taking up the fluorescent label and the "painted" chromosomes can be examined under the microscope.

The method is a modification of fluorescence in situ hybridization (FISH) and is also called **Zoo-FISH**.

Chromosome painting has shown, for example, that large sections of human **chromosome 6** (which includes hundreds of genes

in the major histocompatibility complex (**MHC**) have their counterpart; i.e. homologous genes, in

- chromosome **5** of the chimpanzee
- chromosome **B2** of the domestic cat
- chromosome **7** of the pig
- chromosome **23** of the cow

Comparing DNA Sequences

Proteins are the expression of genes so why not compare the actual gene sequences? There are several advantages:

- DNA is much easier to sequence than protein.
- Genes contain sites that are much freer to change during evolution than protein sequences are. These include:
  - nucleotides that produce **synonymous codons**. For example, even if the amino acid at position 20 in two proteins is the same, the codons for that amino acid might be different in the two species.
  - **Introns** and **flanking sequences**. These regions are relatively free to vary without hurting the final protein product. In other words, these regions of the genome are under much less pressure from natural selection.
- DNA is more stable than protein in the environment. This raises the possibility of doing DNA sequencing on the remains of extinct organisms. Neaderthal remains over 38,000 years old have yielded samples of DNA that were successfully sequenced.

Some of the most informative studies using comparative DNA sequencing have been done with

- **rDNA genes**; that is, the genes encoding the rRNA molecules (usually of the small subunit (**18S** in eukaryotes; **16S** in bacteria) of the ribosome.
- genes on mitochondrial DNA (mtDNA).

In both cases, the genes are present in **multiple copies** making their isolation easier.

Cladistics

Ideally, a system of classification should reflect the genealogies of the organisms. Darwin realized this when he wrote: "our classifications will come, as far as they can be so made, genealogies". A classification based strictly on the rule that all members of a group must have shared a common ancestor more recently than they have with any species outside the group is called cladistics.

This phylogenetic tree or **cladogram** depicts the evolutionary relationships of 4 hypothetical species.

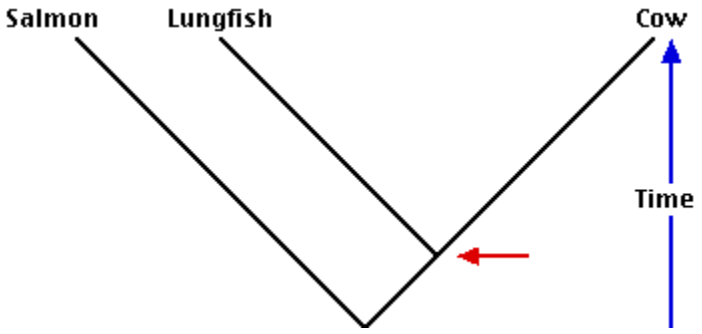


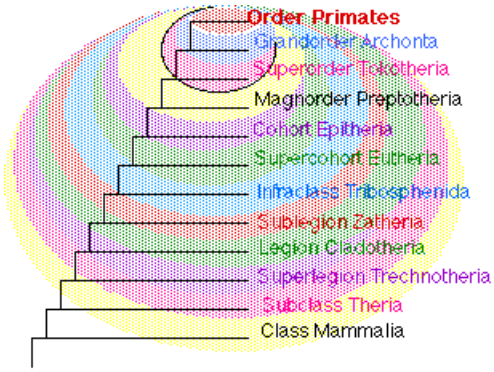
Figure 19.1.1.4 Lungfish - cow cladogram

- They are all descended from an ancestor with 5 traits (1,2,3,4,5) to be used in drawing the tree.
- Over the course of time, 3 speciation events occurred producing the branches.
- During this time, several of the ancestral traits evolved into a modified or derived form; each one indicated by a different color.
- Taxonomists who use cladistic methods have created an extraordinary vocabulary to help them (not necessarily us).
  - Ancestral traits are called **plesiomorphic** (shown here as black numbers).
  - Derived traits are called **apomorphic** (shown here as colored numbers). All the members of a clade must share one or

more apomorphic traits not found in any other species.
    - Derived traits shared by two or more species are called **synapomorphic**. Here species A and B share the synapomorphic trait designated with a blue 3.
    - Ancestral traits shared by two or more species are called **symplesiomorphic**. Here, the trait shown as black 1 is a symplesiomorphic trait retained by all 4 species.
- Note that in comparing the species, the more recent the common ancestor, the more apomorphic traits they share. Thus species **C** and **D** share 4 of the 5 traits but only three (**1**, **2**, and **5**) with species **A** and only two (**1** and **5**) with species **B**.

Even if we reconstruct a precise genealogy and draw a phylogenetic tree to represent it, taxonomic problems may still remain.

1. The species is the only taxonomic category that exists in nature. All higher categories (e.g., genus, family, and order) are purely arbitrary. They are created by taxonomists. For example,
    - Should species **C** and **D** be placed in a single genus with **A** and **B** in another?
    - Or are all four sufficiently closely related that they belong in a single genus?
    - Or are all four so distantly related that they should be placed in separate genera?
    - Note that none of these options (and others besides) violates the fundamental rule that all the members of any one group (or "**clade**") must have had a common ancestor more recent than any they share with species in other groups.



Kingdom	Animal
Phylum	Chordata
Subphylum	Gnathostomata
Class	Mammalia
Order	Primates
Family	Hominidae
Genus	Homo
Species	Homo sapiens

Figure 19.1.1.5 Primate clades

Those taxonomists who are particularly impressed by the differences between species tend to increase the number of higher categories. Those with this bias are known fondly as "**splitters**". "**Lumpers**", those taxonomists who marvel at the uniformities they see among species, tend to create fewer higher categories. Thus, splitters might put each of the 4 species in separate genera while lumpers would put them in a single genus.

2. Classifications based strictly on cladistics are too complex for convenience. In principle, a separate category has to be created for all the branches derived from each node of the tree. The box shows the conventional classification of *Homo sapiens* (in the order **Primates** of the class **Mammalia**). Compare it with the graphic above the box showing a classification of just the primates based more closely on cladistics.

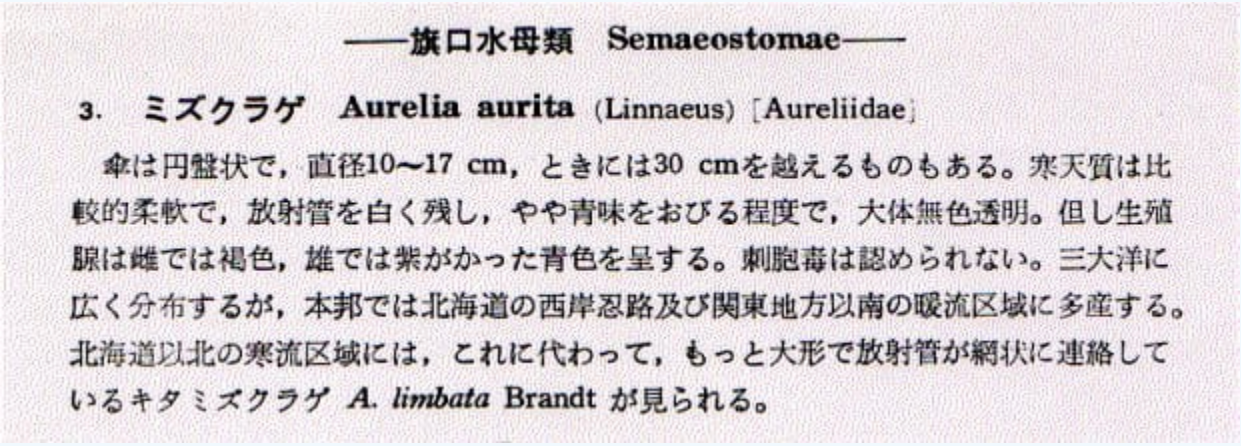
Example

Scientific names. The Swedish naturalist Carolus Linnaeus - the "father of taxonomy" - created the system for naming species that is used by biologists throughout the world. The scientific name of each species consists of two parts:

- the name of the genus to which it is assigned and
- the "specific epithet" which identifies the particular species within the genus.

Latin names were used by Linnaeus, but so many species have been discovered since then that now taxonomists simply coin new words and cast the genus name in the form of a Latin noun and the specific epithet as a Latin adjective. By tradition, both names are printed in italics, and the genus name is capitalized, but not the specific epithet. Note, too, that the characters of the Roman alphabet are always used even by biologists in countries where different characters are used for ordinary purposes.

Here is a description of a common jellyfish as it appears in a Japanese guide to marine life.



——旗口水母類　Semaeostomae——

3.　ミズクラゲ　**Aurelia aurita** (Linnaeus)［Aureliidae］

傘は円盤状で，直径10～17 cm，ときには30 cmを越えるものもある。寒天質は比較的柔軟で，放射管を白く残し，やや青味をおびる程度で，大体無色透明。但し生殖腺は雌では褐色，雄では紫がかった青色を呈する。刺胞毒は認められない。三大洋に広く分布するが，本邦では北海道の西岸忍路及び関東地方以南の暖流区域に多産する。北海道以北の寒流区域には，これに代わって，もっと大形で放射管が網状に連絡しているキタミズクラゲ *A. limbata* Brandt が見られる。

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3. A classification based strictly on evolutionary kinship (cladistics) also may often seem to violate common sense. Thus a phylogenetic tree showing the evolutionary history that gave rise to the salmon (a fish), the lungfish, and the cow requires - according to cladistics - that the lungfish and cow be placed in a **clade** separate from the salmon. Even though the lungfish is a fish, the cow has shared a common ancestor with it more recently than its common ancestor with the salmon. Although it is traditional to classify the lungfish and the salmon together in the class **Pisces** (fishes), and to assign the cow to the class **Mammalia**, this violates the rule of cladistics (so Pisces is said to be a paraphyletic group). The lungfish and the cow with their **apomorphic traits** of internal nostrils and epiglottis are descended from a common ancestor (red arrow) that is also the ancestor of all land-living vertebrates (including ourselves!).

Even Darwin recognized that kinship alone was not always enough for a sound taxonomy so he added a second criterion - degree of similarity - to be used in assigning species to a taxonomic category.

1. Deducing the evolutionary history of animals is particularly difficult because all the 24 or more phyla of **animals** appeared within a short time before and during the Cambrian and have since evolved along separate lines. This means that all the branches on the phylogenetic tree are long and bunched so closely at their base that it is difficult to determine their relationships.
2. **Computer power.** More data would help, but as more data become available, the ability of computer programs to sort out the most likely tree becomes overwhelmed.
3. **Changing rate of evolution.** There is considerable evidence that mutation rates are not steady from branch to branch in phylogenetic trees. Thus a branch based on molecules that have evolved rapidly would seem longer than otherwise.
4. **Back mutations.** These mask the changes that preceded them and make branches look shorter than they should be.
5. **Gene transfer between species.** The recent availability of complete gene sequences for many bacteria have revealed genes that appear to have passed from one group to another rather than having been descended from a common ancestor. Most of these "horizontal" gene transfers are between two different species of bacteria, but the gene sequence of ***Mycobacterium tuberculosis*** reveals 8 genes that it appears to have picked up from its human host! So many horizontal gene transfers have occurred that some bacterial taxonomists despair that a proper phylogenetic tree can ever be deduced for them.
6. **Convergent evolution.** Evolution in which two species from different genealogies come to resemble each other is called convergent evolution and structures that resemble each other superficially (and may serve the same function) are called **analogous**.



Figure 19.1.1.6 Wombat

There are many examples of marsupial mammals in Australia which bear a striking resemblance to placental mammals of Europe and North America. The North American woodchuck or groundhog and the Australian wombat (photo courtesy of the Australian News and Information Bureau), for examples, look superficially to be close relatives. But their similarities are analogous, not homologous, and have arisen as a result of similar selection pressures in similar ecological niches. The wombat has no placenta, cares for its young in a pouch as other marsupials do, and should be classified with them. In fact we are more closely related to the North American woodchuck than the wombat is!

In the language of cladistics, the wombat is placed in a clade with all marsupials because they share the marsupial pouch (an apomorphic trait) but are nonetheless mammals because they, too, have hair (a plesiomorphic trait).

Convergent evolution also occurs at the level of molecules.

Examples:

- Cows and langur monkeys both synthesize a lysozyme that share the same activity, but comparison of their amino acid sequences indicates that each has evolved from a different ancestral molecule.
- Cows and the bacterium *Yersinia* both synthesize a **tyrosine phosphatase** with similar three-dimensional structures around their active site and similar activity. However, each has evolved from a totally different ancestral molecule.
- The bacterium *Bacillus subtilis* synthesizes a serine protease that acts just like those synthesized by mammals but not only has an entirely different primary structure but its three-dimensional structure (tertiary) structure is different as well.
- Representatives of four different orders of insects, orders that last shared a common ancestor 300 million years ago, have independently evolved an identical point mutation in their Na^+/K^+ ATPase which protects it from inactivation by the cardiac glycosides in the plants on which they feed. Link to an illustrated discussion of how this mutation can lead to aposematic coloration and mimicry.

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19.1.2: Protists

What are protists?

- They are **eukaryotes** because they all have a **nucleus**.
- Most have **mitochondria** although some have later lost theirs. Mitochondria were derived from aerobic alpha-proteobacteria that once lived within their cells.
- Many have **chloroplasts** with which they carry on photosynthesis. Chloroplasts were derived from photosynthetic **cyanobacteria** living within their cells.
- Many are unicellular and all groups (with one exception) contain some unicellular members.
- The name Protista means "the very first", and some of the 80-odd groups of organisms that we classify as protists may well have had long, independent evolutionary histories stretching as far back as 2 billion years. But genome analysis added to other criteria show that others are derived from more complex ancestors; that is, are not "primitive" at all.
- Genome analysis also shows that many of the groups placed in the Protista are not at all closely related to one another; that is, the protists do not represent a single clade.
- So we consider them here as a group more for our convenience than as a reflection of close kinship, and a better title for this page would be **"Eukaryotes that are neither Animals, Fungi, nor Plants"**.

The Euglenozoa

Most Euglenozoa are unicellular. Many swim by means of a single flagellum. They are not encased in a cell wall so they are flexible as well as motile. **Euglena** is a typical member of the group (which numbers about 1600 species). Because some members of the group (like Euglena) have chloroplasts, these organisms used to be called "Euglenophytes", but in fact they are neither plants ("phytes") nor animals ("zoa"). Rather — like the other organisms on this page — they are the living descendants of some of the very earliest eukaryotes. ***Trypanosoma brucei***, the cause of African sleeping sickness in humans, is a member of the group. The electron micrograph shows T. brucei as it occurs in the salivary gland of the tsetse fly ready to be injected into the mammalian host when the fly bites. The specimen is 12 µm long.



Figure 19.1.2.1 T. Brucei (by L. Tetley; courtesy of Keith Vickerman)

In Latin America, ***Trypanosoma cruzi***, another member of the group, is the cause of **Chagas disease** in humans.

Ciliates, Sporozoans, and Dinoflagellates: the Alveolates

These three phyla are grouped in a clade called the **alveolates** because they all have a system of saclike structures ("alveoli") on the inner surface of their plasma membrane as well as close homology in their gene sequences.

Ciliates

The ciliates move by the rhythmic beating of their cilia. Although single-celled, some are large enough to be seen with the naked eye. In fact, the tiny parasitic wasp *Megaphragma mymaripenne*, with its tens of thousands of cells (4,600 neurons alone), is

no larger than Paramecium. They feed by sweeping a stream of particle-laden water through a "mouth" and "gullet" and into a **food vacuole**. Undigested wastes are discharged at a permanent site. Fresh water ciliates cope with the continuous influx of water from their hypotonic surroundings by pumping it out with one or more **contractile vacuoles**. Parasitic ciliates, which live in isotonic surroundings, have no contractile vacuole. All of this rightly suggests that although they are unicellular, there is nothing rudimentary about the ciliates. Their single cell is far more elaborate in its organization than any cell out of which multicellular organisms are made. Examples: **Paramecium**, **Stentor**, **Vorticella**, ***Tetrahymena thermophila***.

Sporozoans (Apicomplexa)

The members of this group share an "apical complex" of microtubules at one end of the cell (hence the name that many prefer to the old name of sporozoans). All the members of the phylum are parasites. The genus **Plasmodium** causes **malaria**, one of the greatest scourges of humans. There are 4 species that infect humans of which ***Plasmodium falciparum*** is the most dangerous. Malaria has probably caused more human deaths than any other infectious disease; even today it is estimated to kill a million people a year in the sub-Saharan Africa. The organism is transmitted from human to human through the bite of mosquitoes of the genus **Anopheles**.

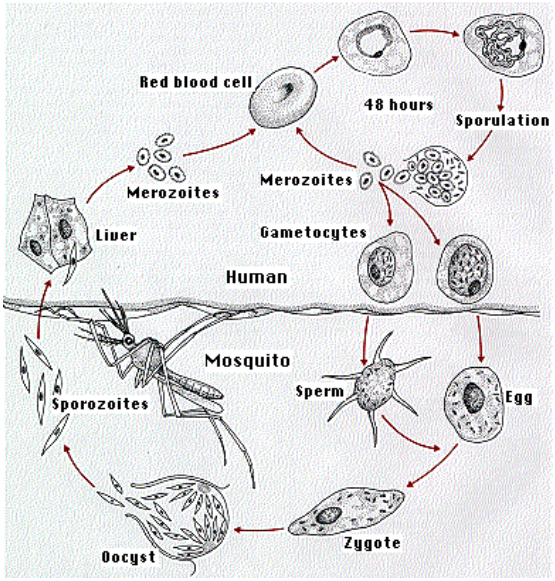


Figure 19.1.2.2 Plasmodium lifecycle

The diagram shows the Plasmodium life cycle.

- The mosquito bite injects **sporozoites** into the human host.
- These invade the liver where they develop into **merozoites**.
- The merozoites invade red blood cells where they reproduce.
- Periodically, they all break out of the red cells together bringing on the chills and fever characteristic of the disease.
- Eventually some merozoites develop into either male or female **gametocytes**.
- These will die unless they are sucked up by the bite of an anopheline mosquito.
- Once in the stomach of the mosquito, the gametocytes form gametes: sperm and eggs.
- These fuse to form zygotes.
- The zygote invades the stomach wall of the mosquito forming thousands of sporozoites.
- These migrate to the salivary gland, ready to be injected into a new human host.
- Most forms of malaria are chronic. The organisms may coexist with their host for years (but cannot complete their life cycle there).

Toxoplasma gondii is another parasitic member of this group. Plasmodium, Toxoplasma, and some of the other members of this group contain a membrane-enclosed organelle called the **apicoplast**. They seem to have inherited it from a common ancestor that acquired it by engulfing a chloroplast.

Dinoflagellates

There are about 1000 species of dinoflagellates. Most are unicellular. Most use chlorophylls **a** and **c.** Unlike most eukaryotes, they lack histones on their chromosomes and have a simpler form of mitosis. They do have the eukaryotic type ("9 + 2") of flagellum (two of them in fact). Occasionally they reproduce explosively, creating poisonous **red tides** that may cause extensive kills of marine fish and make filter-feeding marine animals like clams unfit for human consumption.

The Stramenopiles

These organisms belong to a single clade, the **stramenopiles** (a/k/a heterokonts). There are four members in this group - diatoms, golden algae, brown algae and water molds. The first three members share:

- a yellow-brown pigment (which gives them their color). It is a carotenoid called **fucoxanthin**.
- chlorophylls **a** and **c**
- All four of them (plus a number of other groups not listed) share genes closely-homologous to those in both green and red algae. This suggests that they are all descended from a heterotrophic eukaryotic ancestor that acquired both a green alga and a red alga by a secondary endosymbiosis. (While the water molds no longer are photosynthetic, they still retain both green and red alga genes.)

Diatoms

Diatoms are unicellular. Their cell wall or shell is made of two overlapping halves. These are impregnated with silica and often beautifully ornamented. The photo (courtesy of Turtox) is of ***Arachnoidiscus ehrenbergi*** magnified some 400 times. Diatoms are major **producers** in aquatic environments; that is, they are responsible for as much as 40% of the photosynthesis that occurs in fresh water and in the oceans. They serve as the main base of the food chains in these habitats, supplying calories to heterotrophic protists and small animals. These, in turn, feed larger animals.

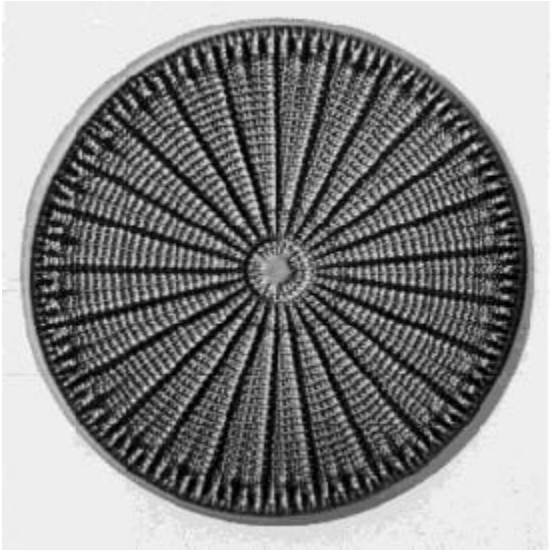


Figure 19.1.2.3 Arachnoidiscus ehrenbergi

Golden Algae (Chrysophyta)

- Most are unicellular.
- Found in fresh water.
- Important producers in some aquatic food chains.
- In low light conditions, may lose their chlorophyll and turn heterotrophic feeding on bacteria and/or diatoms.
- Over 1000 species alive today; many more in the fossil record.

Brown Algae (Phaeophyta)

- The rockweeds and kelps. Some kelps grow as long as 30 meters.
- All are multicellular although without much specialization of cell types.
- Most are found in salt water.
- Used for food in some coastal areas of the world and harvested in the U. S. for fertilizer and as a source of iodine.

Water Molds (Oomycetes)

As their name suggests, water molds were once considered to be fungi. But unlike fungi, the cell wall of water molds is made of cellulose, not chitin. Furthermore, their gene sequences are very different from those of fungi (and most closely related to those of diatoms, golden and brown algae).

Some notable water molds:

- Some species (e.g., **Saprolegnia**, **Achyla**) are parasites of fishes and can be a serious problem in fish hatcheries.
- **Downy mildews** damage grapes and other crops.
- ***Phytophthora infestans***, the cause of the "late blight" of potatoes. In 1845 and again in 1846, it was responsible for the almost total destruction of the potato crop in Ireland. This led to the great Irish famine of 1845–1860. During this period, approximately 1 million people starved to death and many more emigrated to the New World. By the end of the period, death and emigration had reduced the population of Ireland from 9 million to 4 million.
- ***Phytophthora ramorum***, which is currently killing several species of oaks in California.

Red Algae

The red algae are almost exclusively marine. Some are unicellular but most are multicellular. Approximately 6000 species have been identified. They are photosynthetic using chlorophyll **a**. Their closest relatives are the green algae and land plants. Like the cyanobacteria, they use as antenna pigments - **phycoerythrin** (which makes them red) and **phycocyanin.** They do not have the eukaryotic "9+2" flagellum. Some are used as food in coastal regions of Asia. **Agar**, the base for culturing bacteria and other microorganisms, is extracted from a red alga.

Slime Molds (Mycetozoa)

Cellular Slime Molds

The organisms in this group have a complex life cycle during the course of which they go through unicellular, multicellular, funguslike (form spores) and protozoanlike (amoeboid) stages. Thousands of individual amoebalike cells aggregate into a slimy mass - each cell retaining its identity (unlike plasmodial slime molds). The aggregating cells are attracted to each other by the cyclic AMP (cAMP) that they release.

With the exception of one species that causes powdery scab on potatoes, these organisms are of little economic importance. However, their combination of traits makes them of great scientific interest. Molecular phylogenies place them in the same clade as animals (metazoa) and fungi.

Plasmodial (Acellular) Slime Molds (Myxomycetes)

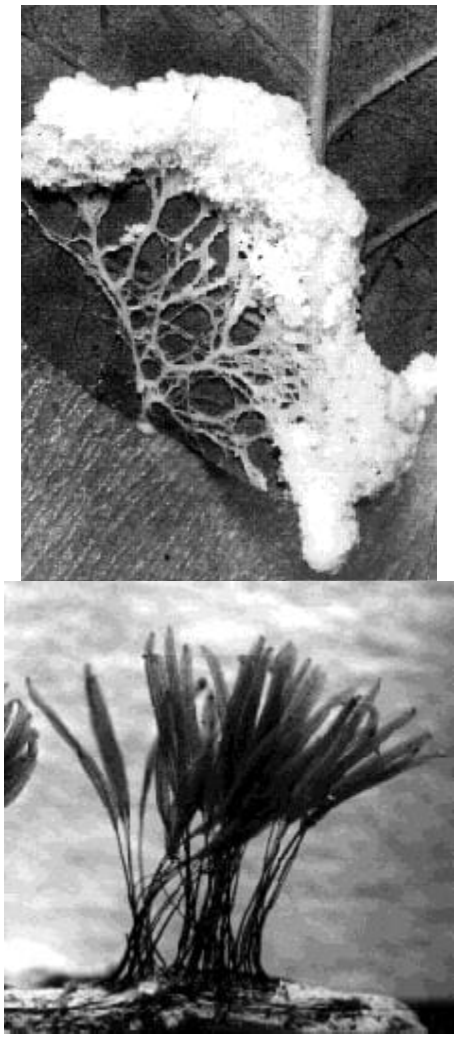


Figure 19.1.2.4 Plasmodial stages of Stemonitis

At one stage in their life cycle, these organisms consist of a spreading, slimy, multinucleate mass called a **plasmodium** that moves slowly over its substrate (e.g., a rotting log) engulfing food and growing as it does so. Eventually, the plasmodium develops stalks that produce and release **spores**. If the spores land in a suitable location, they germinate forming single cells that move by both flagella and pseudopodia. These fuse in pairs and start forming a new plasmodium.

The left photo (courtesy of Prof. I. K. Ross) shows the plasmodial stage of **Stemonitis** just before it formed sporangia. The right photo (courtesy of Turtox) shows the fully developed sporangia of **Stemonitis**.

Physarum polycephalum, another member of this group, is the subject of many laboratory studies.

Protists without typical mitochondria

There are several groups of protists that were long thought to have no mitochondria. However, most (perhaps all) had them in the past. Today, only remnants of their ancestor's mitochondria - called **mitosomes** remain.

Some examples are:

- **Microsporidia**
  - All are unicellular obligate intracellular parasites.
  - Many are pathogenic in insects (one is even marketed commercially as a biocontrol agent).

- Some contaminate drinking water supplies and can cause gastrointestinal upsets in humans. Microsporidia, such as *Encephalitozoon cuniculi*, are a common cause of diarrhea in AIDS patients. *Encephalitozoon cuniculi* has a tiny genome with only 1,997 protein-encoding genes — fewer than many bacteria (e.g., *E. coli* has 4,290). Obliged to live within the cells of its host, it has lost the genes for many important functions (e.g., the citric acid cycle) depending instead on its host.
- Fungi are their closest relatives.

- ***Entamoeba histolytica***.
  - Causes amebic dysentery, the third most common parasitic disease of humans (after malaria and schistosomiasis). Its closest relatives are the slime molds.
- ***Giardia intestinalis*** (also known as *Giardia lamblia*)
  - Frequently encountered in public water supplies contaminated by animal feces. Causes diarrhea in humans. Avoids the host immune response by periodically changing its surface protein coat.

Choanoflagellates

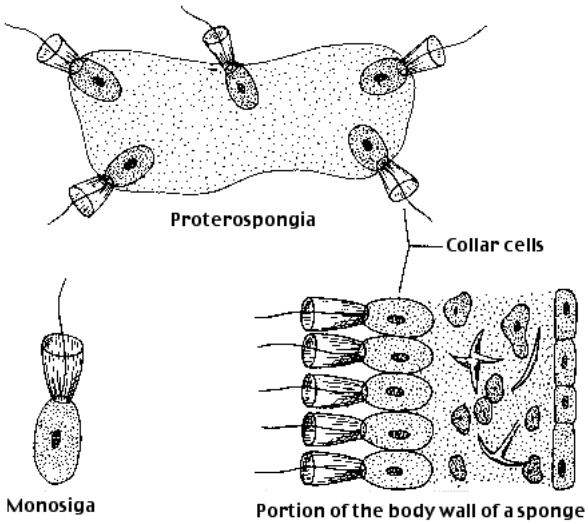


Figure 19.1.2.5 Choanoflagellates

These are single-celled (e.g., Monosiga), aquatic (both fresh water and marine) protists that have a single flagellum surrounded by a collar ("choano" = collar) of microvilli. Some (e.g., Proterospongia) form simple colonies during part of their life. The flagellum is used for swimming and also beats bacteria-containing water through the collar for feeding.

Sponges also use collar cells to filter food from the water. Not only does this suggest a close relationship between the two groups, but other evidence indicates that choanoflagellates are the closest protistan relatives of all animals (metazoa). Although single cells, they express genes for several proteins that are essential to cell-cell interactions in metazoans, such as

- **cadherins** (attach cells to each other)
- **tyrosine kinases** (used in many examples of cell-cell signaling)

What function these proteins have in the choanoflagellates is unknown.

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19.1.3: Ciliates

This page discusses ciliates, complex single-celled protists visible to the naked eye and resembling multicellular organisms. Some biologists propose classifying them as acellular due to their intricate organization. Ciliates have a small diploid micronucleus that contains their genome and a large polyploid macronucleus for gene functions. They reproduce sexually, promoting genetic diversity.

Although single-celled, there is nothing primitive or simple about these protists. Not only are they large for single cells (some can be seen by the unaided eye), but they contain organelles that parallel in function the organs of multicellular creatures. In fact, some biologists consider the ciliates to be **acellular** (not cellular) rather than unicellular in order to emphasize that their "body" is far more elaborate in its organization than any cell out of which multicellular organisms are made.

Ciliates have:

- At least one small, diploid (2**n**) **micronucleus**. It contains the entire genome but is not active in gene transcription.
- A large, polyploid **macronucleus** that contains the active genes that run the cell.

Sexual reproduction in Ciliates

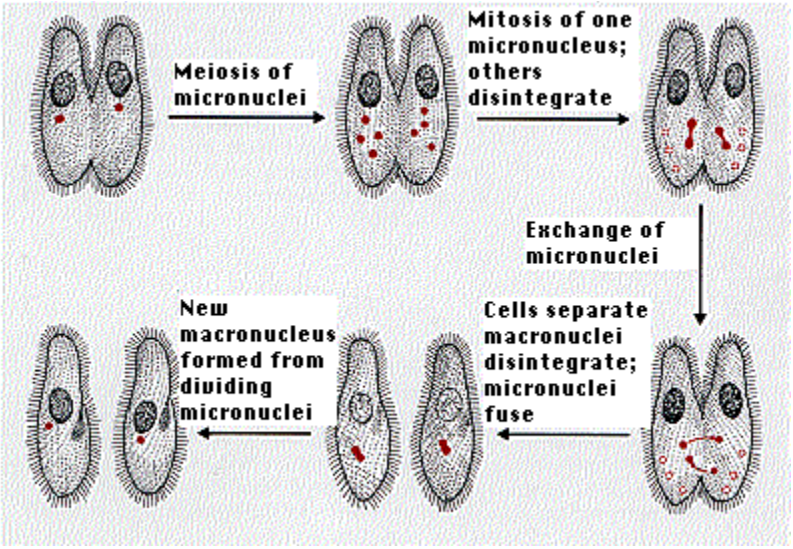


Figure 19.1.3.1 Paramecium reproduction

- Two parents come together and two parents separate. What kind of reproduction is that? you may well ask.

  But the process they have been through is the very essence of sexual reproduction - genetic recombination. The "offspring" are not the same as the parents. They are new individuals and their macronucleus will soon reflect that fact.

  Ciliated protozoans have been the source of several important discoveries in biology, for example:
- The first ribozymes were found in *Tetrahymena thermophila*.
- Telomerase was also discovered in *Tetrahymena thermophila*.

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19.1.4: Volvox

This page discusses Volvox, a multicellular green algae genus that reproduces both asexually, through gonidia from a dying parent, and sexually, with male and female gonidia forming sperm clusters and eggs respectively. Fertilized eggs develop into zygotes that withstand harsh conditions. Additionally, it mentions that Volvox carteri's genome has 14,560 protein-encoding genes, comparable to Chlamydomonas reinhardtii, but with extra genes for its extracellular matrix.

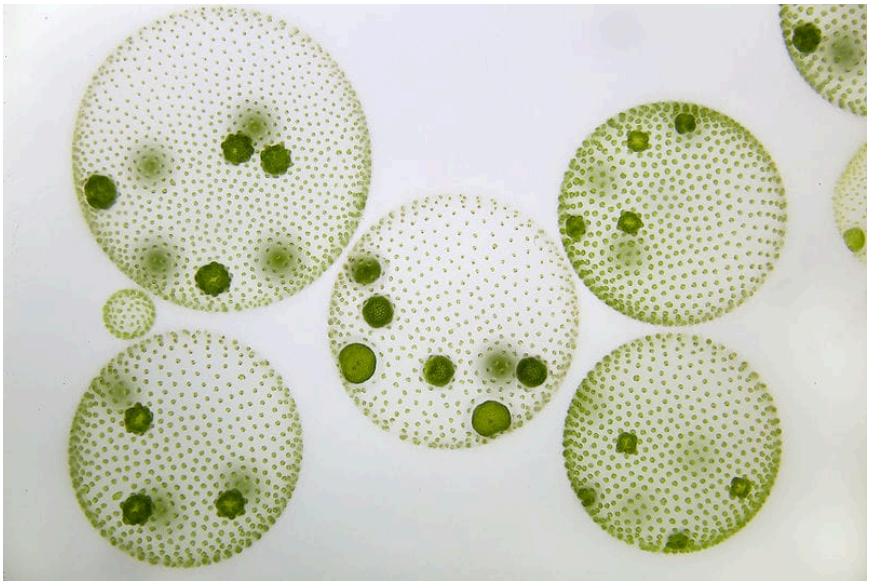


Figure 19.1.4.1: Volvox is a genus of multicellular green algae. by http://www.mikro-foto.de (CC-SA-BY Frank Fox)

Volvox Reproduction

Volvox can reproduce both asexually and sexually. In asexual reproduction, the gonidia develop into new organisms that break out of the parent (which then dies). In sexual reproduction, the presence of an inducing chemical causes

- The gonidia of the males to develop into clusters of sperm.
- The gonidia of the females to develop into new spheres each of whose own gonidia develops into a pair of eggs.
- The sperm break out of the male parent and swim to the female where they fertilize her eggs.
- The zygotes form a resting stage that enables Volvox to survive harsh conditions.

The genome of *Volvox carteri* consists of 14,560 protein-encoding genes - only 4 more genes than in the single-celled *Chlamydomonas reinhardtii*! Most of its genes are also found in *Chlamydomonas*. The few that are not encode the proteins needed to form the massive extracellular matrix of *Volvox*.

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19.1.5: Diversity and Evolutionary Relationships of the Plants

Evolution and Classification

The organisms we call plants are assigned to a single clade; that is, a natural grouping based on the belief that they have all evolved from a common ancestor more recent than any shared with other organisms. Among the criteria for doing this are:

- their shared use of the photosynthetic pigments **chlorophyll a** and **chlorophyll b**
- the similarities in the nucleotide sequences of the genes encoding both their small subunit (18S) and large subunit (25S) **ribosomal RNA**
- their shared cellulose cell wall

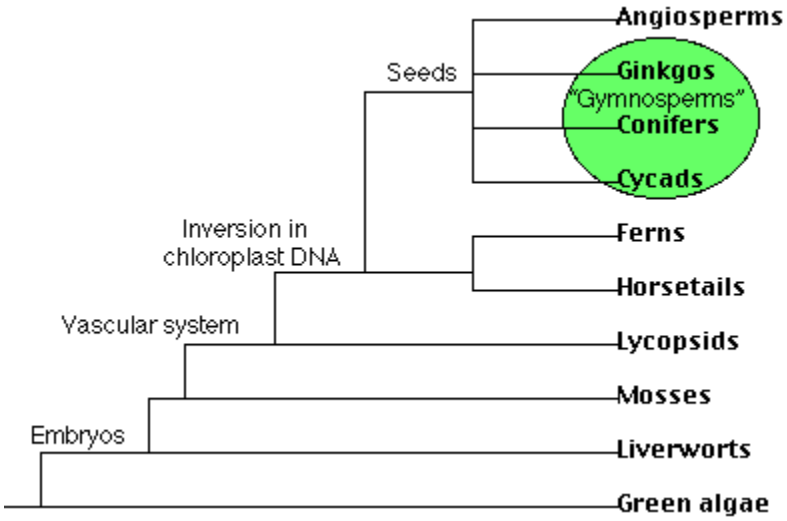


Figure 19.1.5.1 Plant clade

We shall examine here a selection of the most prominent groups.

Green Algae

The ancestors of these organisms were the most primitive members of the clade. In other words, organisms that we would put in this division were probably the ancestors of all the other plants. There are some 7000 species living today. They include:

- microscopic, unicellular forms like **Chlamydomonas**
- colonial forms like the filamentous **Spirogyra**
- multicellular forms like **Volvox** and **Ulva**, the sea lettuce

Although some of the multicellular forms are large, they never develop more than a few types of differentiated cells and their fertilized eggs do not develop into an embryo.

Green algae are an important source of food for many aquatic animals. When lakes and ponds are "fertilized" with phosphates and nitrates (e.g., from sewage and the runoff from fertilized fields and lawns), green algae often form extensive algal "blooms".

Liverworts and Mosses (Bryophytes)

These are fairly simple plants that do produce a number of differentiated cell types and whose fertilized egg develops into a distinct embryo.

However, they have neither vascular tissue (xylem and phloem) nor woody tissue and thus never grow very large. Some 16,000

living species are known. Most grow in moist places.

Lycopsids (Lycophytes)

Prominent members of this group are often called **club mosses**. They are not mosses at all, but **vascular plants** with **xylem** and **phloem** running through their roots, stems, and leaves. The leaves are quite simple and small with their vascular tissue in a single, unbranched vein. The "club" of their name comes from the appearance of their spore-forming structures called **strobili**. Club mosses are also sometimes called "ground pines", but they are not pines either. The photo shows ***Lycopodium obscurum***.



Figure 19.1.5.2 Club moss

About 1000 species of lycopsids exist today. All are small (those in the photo stand about 8 in. [20 cm] tall), but it was not always so. Fossil lycopsids in the Mississippian and Pennsylvanian periods (the so-called Carboniferous era) reached heights of 100 feet (30 meters). Their remains contributed to the formation of coal.

Chloroplast Genes

Chloroplasts (as well as mitochondria) have their own genome. The diagram (based on the work of Ohyama, K. et al., **Nature** 322:572, 1986 and Linda A. Raubeson and R. K. Jansen, **Science** 225:1697, 1992) shows the genome of the first chloroplast DNA to be sequenced, that of the liverwort ***Marchantia polymorpha***. It contains 121,024 base pairs encoding 128 genes. The short lines indicate a few of the tRNA genes, some of which are labeled.

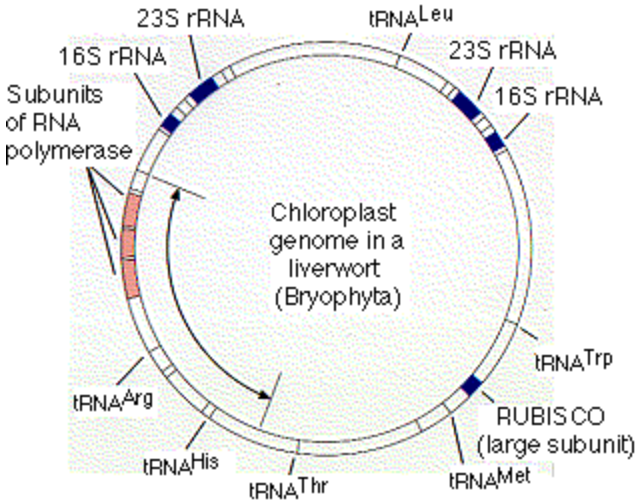


Figure 19.1.5.3 Chloroplast

The order of the genes between the arrows (~6:30 to ~10:00) is also found in the **lycopsids**. But in **all other vascular plants**, this region is **inverted** and the order of the genes is precisely reversed. This provides further evidence that the other vascular plants we shall examine below, the

- horsetails
- ferns
- gymnosperms
- angiosperms

belong to a separate **clade**.

Horsetails

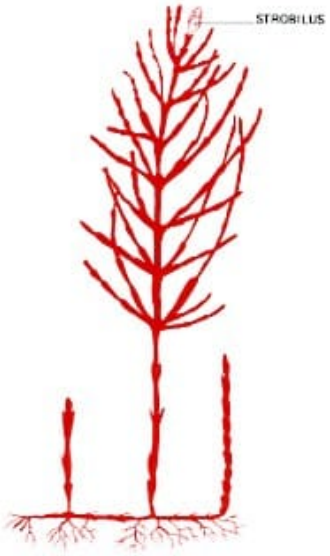


Figure 19.1.5.4 Horsetails

(Classified as **Equisetopsida** although many botanists prefer the older term **Sphenopsida**.)

The common name comes from the characteristic pattern of branching: whorls or rings of branchlets arising from an above-ground shoot. The shoot develops each season from an underground stem (rhizome). Horsetails often grow in sandy places and incorporate silica in their stems. This gives them an abrasive quality which caused them to once be used for cleaning pots and pans, which gave rise to another common name: scouring rush.

Only one genus, **Equisetum**, containing about 25 species, survives today. However, many other, much larger, species were dominant features of the Carboniferous and, like the early lycopsids, contributed to the formation of coal.

The drawing is of *Equisetum palustre*, a common horsetail. Spores are formed in the strobilus.

Ferns

(Assigned to the **Pteridopsida** although some botanisits prefer Filicopsida.)

Some 15,000 species of ferns live on earth today. Many of these are found in the tropics where some — the "tree ferns" — may grow to heights of 40 ft (13 m) or more. The ferns of temperate regions are smaller. They are usually found in damp, shady locations. Their stems — called **rhizomes** — as well as their roots grow underground and are perennial. Their leaves, called **fronds**, grow up from the rhizome each spring.

Seed Plants (Spermatophytes)

Gymnosperms

Fossil from the Devonian period reveal fernlike plants that were **heterosporous**; that is, produced two kinds of spores: microspores (male) and megaspores (female). The megaspores were not released from the parent sporophyte. Fertilization took place within the tissue of the parent sporophyte thus freed from dependence on surface water. However, the necessity for the microspores to be carried from one plant to another in order to reach the female gametophyte robbed them of their value as agents of dispersal. This function was taken over by **seeds -** dormant, protected, embryo sporophytes.

The **seed ferns**, as these plants are called, were among the earliest gymnosperms. Although seed ferns are now extinct, some of their living descendants, the **cycads**, resemble them closely. Cycads reveal their ancient lineage by the fact that after the microspore reaches the ovule, it liberates a ciliated sperm which, swimming in moisture supplied by the parent sporophyte, reaches the egg. **Ginkgos** are also gymnosperms that use motile sperm.

Conifers

These gymnosperms get their name from their **cones**: male cones in which the in which microspores develop and female cones in which megaspores develop. The microspores develop into **pollen grains** that are carried by the wind to the female cones. Here each germinates into a **pollen tube** which grows into the tissues of the female cone until it reaches the vicinity of the egg. (In pines, this may take a year.) Then the tube ruptures and a **sperm nucleus** fuses with the egg to form the zygote.

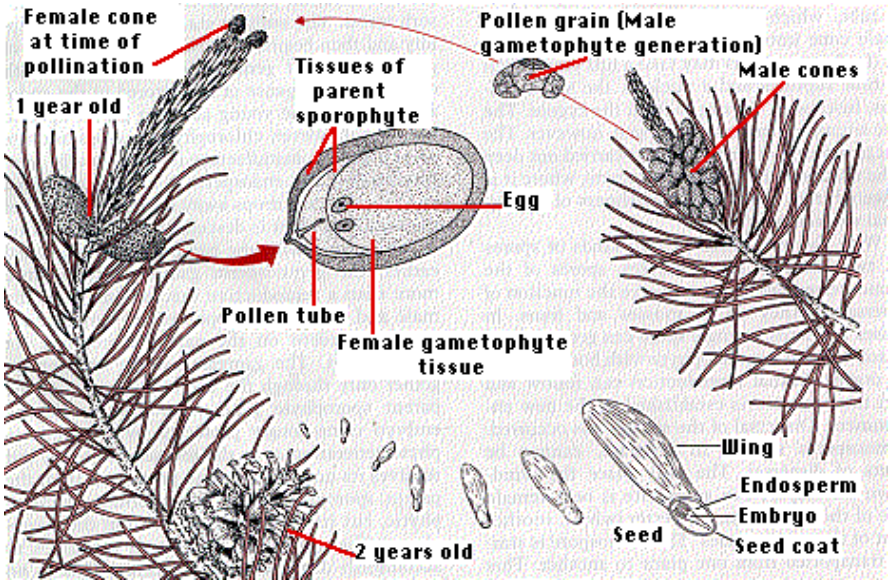


Figure 19.1.5.5 Pine lifecycle

After fertilization, the zygote develops into a tiny embryo sporophyte plant. There are approximately 630 species of living conifers. They include the pines, spruces and firs. Conifers include the largest and the oldest of all living organisms. One redwood (genus **Sequoia**) growing in California is almost 400 feet (122 meters) high. Bristlecone pines growing in the mountains of eastern California are more than 4000 years old.

Although most conifers are evergreen, their leaves are modified as "needles", and these reduce snow load and transpiration during the winter in the harsh high-latitude climates where conifers are the dominant species of plants. But by retaining their needles during the winter, conifers are ready to begin photosynthesis immediately upon the return of spring.

Coniferous forests are of great economic importance producing lumber for building and pulp for paper making.

Angiosperms

Although angiosperms appear in the fossil record in Jurassic deposits, it was not until the end of the Mesozoic era that angiosperms became the dominant plants of the landscape. That they dominate the earth's flora today is clear: there are some 260,000 species of living angiosperms; the rest of the plant kingdom includes only some 47,700 species. Currently, the angiosperms are classified in some 54 **orders** (The names of the orders end in ..**ales**, e.g., Arabidopsis is in the order Brassicales.). Each order contains from one to several dozen **families** (Family names end in ..**aceae**, e.g., Arabidopsis is in the family Brassicaceae).

Monocots and Dicots

Of over 400 families of angiosperms, some 80 of them fall into a single clade, called monocots because their seeds have only a single cotyledon. The remainder are the dicots whose seeds have two cotyledons. The large majority of these occupy a single clade called the **eudicots**.

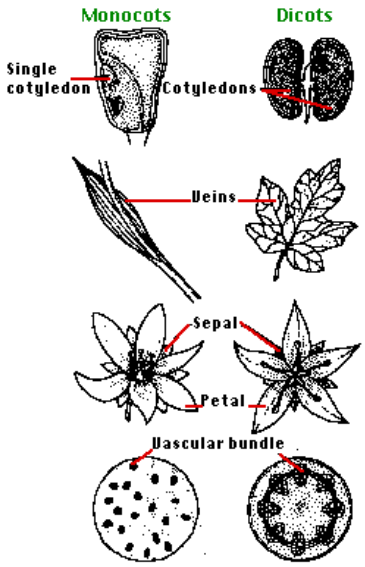


Figure 19.1.5.6 Monocot vs dicot

Monocot traits:

- a single cotyledon in their seed
- parallel venation in their leaves
- petals and sepals in 3s or some multiple thereof
- vascular bundles scattered randomly throughout the stem

Monocots include:

- palms (Arecaceae)
- orchids (Orchidaceae)

- yams, sweet potatoes (Dioscoreaceae)
- lilies, onion, asparagus (Liliaceae)
- bananas (Musaceae)
- and all the **grasses** (Poaceae), which include many of our most important plants such as
  - corn (maize)
  - wheat
  - rice
  - and all the other cereal grains upon which we depend so heavily for food as well as
  - sugar cane and bamboo

Dicot traits:

- two cotyledons in their seeds
- netted venation in their leaves
- petals and sepals in 4s, 5s, or some multiple thereof
- vascular bundles in the stem arranged in a radial pattern like spokes of a wheel.

Here is a selection of eudicots.

Family	Examples
Anacardiaceae	poison ivy, cashews, pistachios
Asteraceae	asters and all the other composite flowers
Brassicaceae	cabbage, turnip; Arabidopsis, and other mustards
Cactaceae	cacti
Cucurbitaceae	squashes
Euphorbiaceae	cassava (manioc)
Fabaceae	beans and all the other legumes
Fagaceae	oaks
Linaceae	flax (source of linen)
Malvaceae	cotton
Oleaceae	olives, ashes, lilacs
Rosaceae	roses, apples, peaches, strawberries, almonds
Rubiaceae	coffee
Rutaceae	oranges and other citrus fruits
Solanaceae	potato, tomato, tobacco
Theaceae	tea
Vitaceae	grapes

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19.1.6: Arabidopsis Thaliana - A Model Organism

Arabidopsis Thaliana has become to plant biology what *Drosophila melanogaster* and *Caenorhabditis elegans* are to animal biology. Arabidopsis is an angiosperm, a dicot from the mustard family (*Brassicaceae*). It is popularly known as thale cress or mouse-ear cress. While it has no commercial value - in fact is considered a weed - it has proved to be an ideal organism for studying plant development.



Figure 19.1.6.1: *Arabidopsis Thaliana*. Photo courtesy of Nicole Hanley Markelz of the Plant Genome Research Outreach Program at Cornell University.

Some of its advantages as a model organism:

- It has one of the smallest genomes in the plant kingdom: 135 x 10^6 base pairs of DNA distributed in 5 chromosomes (2n = 10) and almost all of which encodes its 27,407 genes.
- Transgenic plants can be made easily using *Agrobacterium tumefaciens* as the vector to introduce foreign genes.
- The plant is small - a flat rosette of leaves from which grows a flower stalk 6–12 inches high.
- It can be easily grown in the lab in a relatively small space.
- Development is rapid. It only takes 5– 6 weeks from seed germination to the production of a new crop of seeds.
- It is a prolific producer of seeds (up to 10,000 per plant) making genetics studies easier.
- Mutations can be easily generated (e.g., by irradiating the seeds or treating them with mutagenic chemicals).
- It is normally self-pollinated so recessive mutations quickly become homozygous and thus expressed.

  Other members of its family cannot self-pollinate. They have an active system of **self-incompatibility**. Arabidopsis, however, has inactivating mutations in the genes — ***SRK*** and ***SCR*** **-** that prevent self-pollination in other members of the family.

- However, Arabidopsis can easily be cross-pollinated to do genetic mapping and produce strains with multiple mutations.

Many of the findings about how plants work - described throughout these pages - were learned from studies with Arabidopsis.

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19.1.7: Fungi

Some 100,000 species of fungi have been identified, but the true number is probably larger.

Characteristics of Fungi

Most fungi grow as tubular filaments called **hyphae**. An interwoven mass of hyphae is called a **mycelium**. The walls of hyphae are often strengthened with **chitin**, a polymer of **N-acetylglucosamine**. The linkage between the sugars is like that of cellulose and peptidoglycan and produces the same sort of structural rigidity.

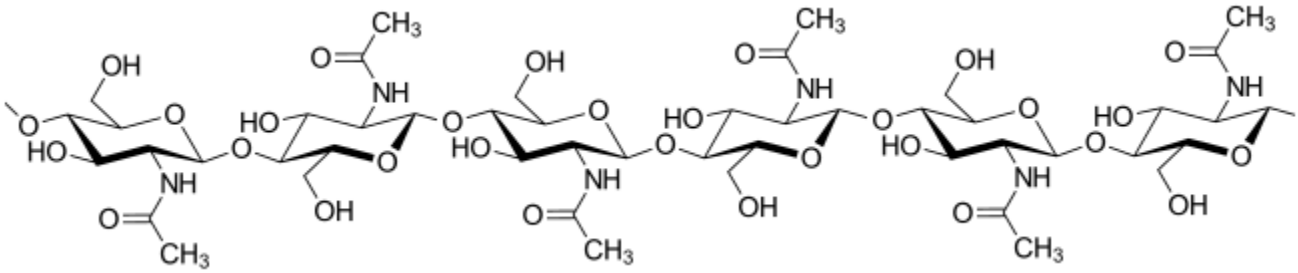


Figure 19.1.7.1 Chitin

Fungi disperse themselves by releasing spores, usually windblown. Fungal spores are present almost everywhere (and are a frequent cause of allergies). Spores of the wheat rust fungus have been found at 4000 m in the air and more than 1450 km (900 miles) from the place they were released. No wonder then that most fungi are worldwide in their distribution.

Fungi are heterotrophic. Some live as saprophytes, getting their nourishment from the surroundings (often having first digested it by secreting enzymes). They perform a crucial role in nature by decomposing dead organisms and releasing their nutrients for reuse by the living. Some live in a mutually beneficial symbiotic relationship with another organism, often a plant. The association of fungus and plant root is called a **mycorrhiza**. Some 80% of land plants benefit from symbiotic mycorrhiza. The plant benefits by more-efficient mineral (especially phosphorus) uptake and the fungus benefits by the sugars translocated to the root by the plant.



Figure 19.1.7.2 Indian pipe

Mycorrhizal fungi may also form conduits for nutrients **between** plant species. The colorless, and hence heterotrophic Indian pipe (*Monotropa uniflora* - pictured on the right) is an angiosperm that must secure all its nourishment from mycorrhizal fungi that are

attached at the same time to the roots of some autotrophic plant such as a pine tree. Radioactive carbon administered to the pine (as CO_2) soon turns up in carbohydrates in nearby Indian pipes.

Some fungi are parasitic, causing serious damage to their host (a few examples are given below). Some fungi are both. *Metarhizium robertsii* is a soil fungus that lives symbiotically with plants but parasitizes (and kills) soil insects. Its hyphae penetrate both the roots of the plant and the corpse of the insect. It has been demonstrated that nitrogen released by the decaying insect is transported by the fungus to the plant. (See S. W. Behie, P. M. Zelisko, M. J. Bidochka in *Science*, 22 June 2012.) *Metarhizium* is an ascomycete.

Ascomycetes

Ascomycetes produce two kinds of spores: asexual spores called **conidia** and **ascospores** produced following sexual reproduction. Four or eight ascospores develop inside a saclike ascus (the group is commonly called sac fungi). Some notable examples include:

- ***Saccharomyces cerevisiae*** one of the **budding yeasts**. It ferments sugar to **ethanol** and **carbon dioxide** and thus is used to make alcoholic beverages like beer and wine, to make ethanol for industrial use and in baking (it is often called **baker's yeast**). Here, it is the carbon dioxide that is wanted (to make bread and cakes "rise" and have a spongy texture). Yeast is also used in the commercial production of some vitamins and in the production - using recombinant DNA technology - of some human therapeutic proteins.
- ***Neurospora crassa***, another favorite "model" organism in the laboratory.
- The fungal partner in most **lichens** is an ascomycete.
- Powdery mildews that attack ornamental plants
- The chestnut blight, which in a few decades killed almost all of the mature American chestnut trees in the Appalachians of North America.
- The Dutch elm disease, which has killed many of the American elms in the United States.
- ***Pneumocystis jirovecii***, which is a major cause of illness in immunosuppressed people, e.g., patients with AIDS.
- The **truffle** and the **morel**, both highly-prized food delicacies. Truffles establish a symbiotic relationship with the roots of such trees as oaks.



Figure 19.1.7.3 Truffles. (Public Domain).

Lichens

Lichens are fungi that live in a symbiotic association with an autotrophic green alga or cyanobacterium (the "photobiont") or - in some cases - both. The fungal partner (the "mycobiont") in most lichens (98% of them) is an ascomycete. Zygomycetes make up the remainder. The relationship is often characterized as mutualistic; that is, both partners benefit. But recent evidence (e.g. in British soldier) suggests that while the fungus is dependent on its autotrophic partner, the photobiont is often perfectly content

to live alone. Recently many lichens have been found to harbor a third partner, a single-celled basidiomycete. Its function remains to be discovered.

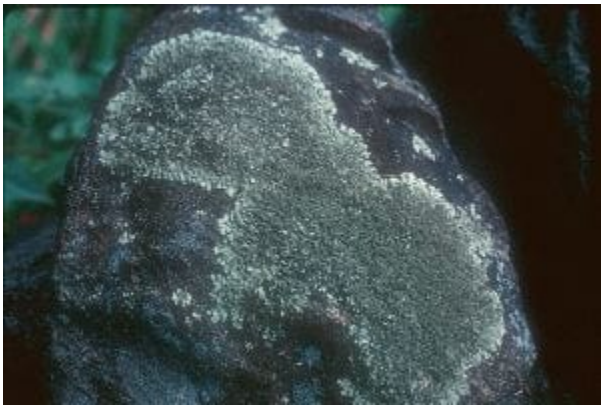


Figure 19.1.7.4 Lichens

Lichens secrete a variety of unusual chemicals; some of these probably assist in the breakdown of rock substrates like the one shown here.

The British Solder

The below image is of the colorful lichen called British soldier. The fungus is ***Cladonia cristatella***, an ascomycete. Its name is the name given to the lichen. The photobiont is *Trebouxia erici*, a green alga. It is found in many other lichens as well, and also can be found growing independently. The algal cells eventually are killed by the fungus, but are continuously replaced by new ones. So the relationship in this lichen is one of controlled parasitism rather than mutualism.



Figure 19.1.7.5 British soldier

The red cap produces the spores of the fungus, but these alone cannot form new lichens. Other structures (e.g., soredia), containing both partners, are needed to disperse the lichen to new locations. Some lichens release only fungal spores. These mycobionts depend for their continued survival on finding an acceptable photobiont released from other lichens. Phylogenetic trees, based on both ribosomal RNA genes and many protein-coding genes, as well as **fossils** indicate that lichens have been present on the earth for at least 600 million years.

Today about 14,000 species of fungi form lichens. Lichens are extremely sensitive to air pollution. One of the best contemporary examples of evolutionary adaptation is the change in coloration of the peppered moth as the lichens in its habitat declined

because of air pollution and then returned when air quality controls were put in place. Some modern fungi (e.g., ***Penicillium chrysogenum***, the source of the antibiotic penicillin) appear to have evolved from lichen-forming ancestors — abandoning their original symbiotic way of life.

Basidiomycetes

Basidiomycetes include **mushrooms**, shelf fungi, puffballs, rusts, and smuts. They are dispersed by spores borne at the tips of **basidia** (giving rise to the name for the group). Mushrooms are masses of interwoven hyphae growing up from the main mass of the mycelium growing underground. The basidia develop on the undersides and release their spores (four from each basidium) into the air.



Figure 19.1.7.6

A single mycelium may expand outward year after year as its hyphae grow into new terrain. In some species, mushrooms are sent up once a year at the periphery producing a circle known since medieval times as a "fairy ring".

Some notable basidiomycetes:

- ***Armillaria bulbosa***. A single specimen in northern Michigan (USA) was found to have spread over 37 acres (15 hectares) of the forest floor. RFLP analysis of samples taken from many different locations within this area showed that all the samples were from a single clone. Assuming the normal rate of vegetative growth for this species, it must have taken 1500 years to spread to that size.
- the cultivated, **edible mushroom** that finds its way into pizza, soups, etc.
- ***Amanita muscaria***. Forms a beautiful mushroom but deadly when eaten.
- **Smuts**. Parasites of important crops like wheat, oats, and rye.
- Rusts. Some, such as
  - **wheat black stem rust** (*Puccinia graminis*) and
  - **white pine blister rust**

  are serious pests. Both have complicated life cycles during which they pass through a second plant host (barberry plants for wheat black stem rust, gooseberries or wild currants for the white pine blister rust).

Zygomycetes

All the fungi assigned to this group (which probably does not represent a single clade) form spores in a **sporangium**. Some notable examples:

- the bread mold, ***Rhizopus stolonifera***
- ***Rhizopus oryzae***, used to make sake, the rice wine of Asia. Can also infect humans, especially if they are immunosuppressed (e.g., AIDS patients, transplant recipients).
- Another species of Rhizopus is used in the commercial production of glucocorticoids.

- Many mycorrhizal fungi belong to this group.

Chytrids

This small group (~1000 species) is thought to be the most primitive of the fungi. Unlike all the other fungi, its members produce flagellated gametes (for sexual reproduction) and flagellated zoospores (for asexual reproduction). They are mostly aquatic.Two species are responsible for the recent worldwide decline in amphibian populations (frogs, toads, and salamanders).

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19.1.8: Yeast

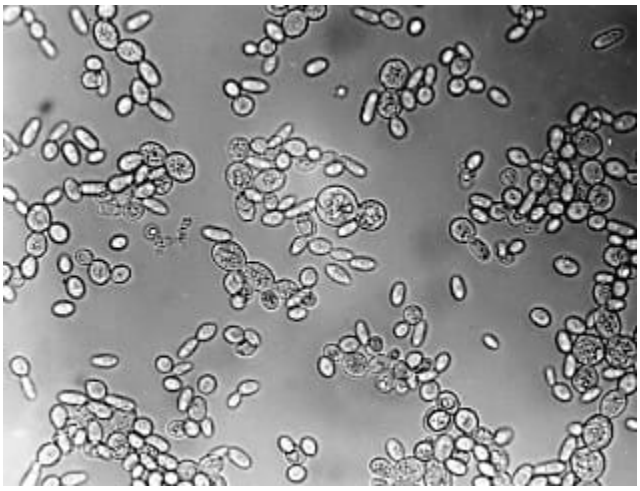


Figure 19.1.8.1 Yeast

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19.1.9: Barcoding

Barcoding is the term applied to a technology that is being developed to speed the identification of specimens of living things. So far, identification and classification of animals has progressed furthest. Although each individual in most species has a unique genome sequence, the differences between individuals of one species are much smaller than the differences between individuals of different species. Thus determining the genome sequence of a specimen should enable it to be positively classified if the sequences of other members of its species are already in a database.

However, sequencing entire genomes of animals is an enormous undertaking. (Most mammals have some 3 billion base pairs of DNA.) A more practical approach is to settle on the sequence of a single gene that is found in all animal life. The one that has been chosen for animals is the gene, *COI*, encoding the largest subunit of cytochrome c oxidase.

Advantages

- It is a mitochondrial gene and thus each cell has hundreds-to-thousands of copies of it as opposed to only two copies of each of its nuclear genes.
- It has no introns (in animals).
- Thanks to the redundancy of the genetic code, it can mutate quite freely, especially in the third position of its codons.
- Furthermore mitochondrial gene sequences vary more between related species than their nuclear genes do. For example, while the sequence differences between the nuclear genes of humans and chimpanzee is only about 1%, the difference between their mitochondrial gene sequences is some 9%.
- Within a species, however, there is little variation from specimen to specimen in their mitochondrial gene sequences.

Procedure

- The mitochondrial DNA is extracted.
- A fragment from the 5' end of *COI* (~648 base pairs) is isolated (the entire gene has ~1500 base pairs).
- The fragment is amplified by PCR using readily-available primers.
- The sequence is determined and compared with those already in a database.

Early Results

Barcoding analysis of several hundred different birds has shown that barcode results usually reflect the species identification based on more conventional criteria. However, a few cases have arisen where:

- specimens thought to belong to the same species differ substantially in the *COI* sequence and thus probably represent convergent evolution.
- specimens thought to belong to different species have similar *COI* sequences and thus are probably local variants of what is actually a single species.

Looking Ahead

- Development of appropriate barcoding genes for plants (whose *COI* genes vary little) and fungi (whose *COI* genes are interrupted by introns). One promising candidate for plants is the chloroplast gene *rbcL* which encodes the large subunit of RUBISCO.
- Development of hand-held sequencers that can barcode the DNA of a specimen in the field.

Promoting the development of barcoding is The Consortium for the Barcode of Life (CBOL). Their home page (link below) provides other links describing goals, methods, achievements, etc. As of this writing, barcodes for over 112,000 species have been entered in databases.

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19.1.10: Invertebrates

The Origin and Evolution of Animals (Metazoa)

We do not yet know from what group(s?) of eukaryotes the animals evolved. It occurred in Precambrian times. Before the Cambrian was far along, most of the animal phyla had appeared. So each of the phyla described in this section has had a long, independent history. The rapid (geologically speaking!) diversification of the animals has made it difficult to establish the genealogical relationships between them — even using molecular data. Our best guesses are shown in the *cladogram* below..

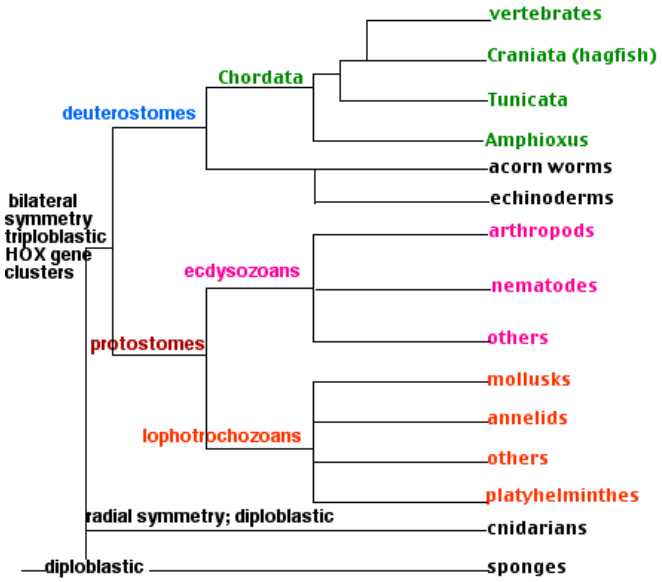


Figure 19.1.10.1 Invertebrates

Sponges (Phylum Porifera)

Sponges are sessile, spending their lives anchored to a solid surface underwater. Most are marine although some live in fresh water. **Diploblastic**; that is, the body wall is made of **two layers** of cells with a jellylike **mesoglea** between them. The body wall is perforated with pores (hence the name Porifera) through which water containing food particles is filtered. The water is drawn in through the pores by **collar cells** like those found in choanoflagellates. Some sponges can process a volume of water more than 100,000 times their own volume in the course of a day!

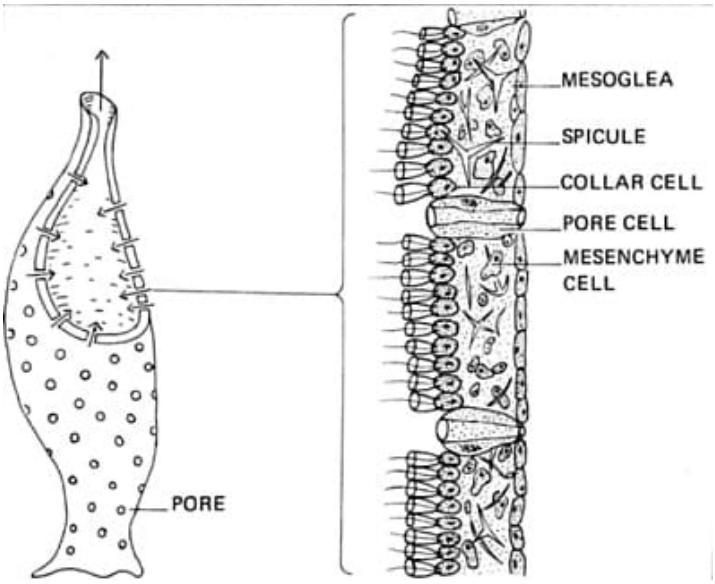


Figure 19.1.10.2 Sponge

Sponges are dispersed by small, free-swimming larvae. There are about 10,000 species known. Sponges are probably the most ancient of today's invertebrates, their fossils appear in the geological record as far back as 635 million years. Despite their simple body plan, sequencing shows that their genome (> 18,000 genes) contains many genes homologous to those found in much more complex animals.

Cnidarians (Phylum Cnidaria)

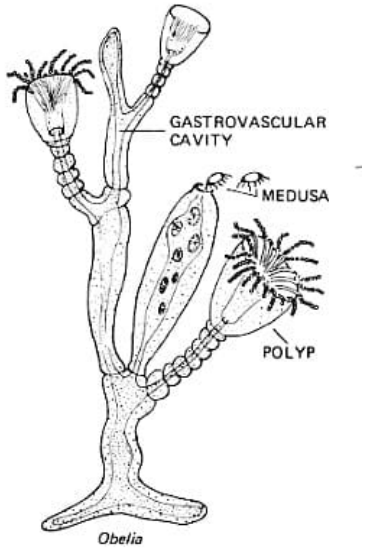


Figure 19.1.10.3 Obelia

Characteristics:

- **diploblastic**; **two layers** of cells - ectoderm and endoderm - with a jellylike **mesoglea** between them;
- predominantly **radial symmetry**: body parts (e.g., tentacles) arranged in whorls. However, in some sea anemones, there is only one plane through the tubular body that divides it into two mirror-image halves; thus revealing bilateral symmetry.
- **cnidoblasts**: specialized cells that secrete a stinging capsule called a **nematocyst**.
- Food is taken through a mouth into the **gastrovascular cavity**. The cavity is also called a **coelenteron** and for many years the name of this phylum was **Coelenterata**. There is no anus.
- Sexual reproduction produces a free-swimming, ciliated larva called a **planula**.

- The phylum contains about 10,000 species distributed in 3 classes:
  - **Hydrozoa** Although the freshwater hydra is a much-studied representative, it is not typical of the class. Most members are
    - marine
    - colonial
    - produce two body forms: the sessile **polyp** (like the hydra) and the free-floating **medusa** (which disperses the species)
  - **Scyphozoa** Jellyfishes (the medusa stage is dominant). The jelly of the medusa is a much-enlarged mesoglea.
  - **Anthozoa** Sea anemones and corals. Have only the polyp stage.

Bilaterians

All the remaining groups of animals belong in a clade whose members share:

1. **bilateral symmetry** (hence the name); that is, **dorsal-ventral** and **left-right** axes
2. **triploblastic** (three tissue layers: **ectoderm**, **mesoderm**, **endoderm**)
3. HOX genes in one or more clusters with the genes within a cluster arranged in the same order as the body parts they affect.

The bilaterians contain two clades, the **protostomia** and the **deuterostomia**.

Protostomia vs. Deuterostomia

Long before the days of genome analysis, taxonomists were convinced of a fundamental division in the animal kingdom between the protostomes ("first mouth") and the deuterostomes ("second mouth").

Protostomia	Deuterostomia
Blastopore forms future mouth (in most groups).	Blastopore forms future anus. Mouth forms later.
Few HOX genes for the posterior	Multiple HOX genes for the posterior
Spiral cleavage of Lophotrochozoan embryos	Perpendicular cleavage planes in embryo
Early cleavage cells committed; no identical twins	Early cleavage cells totipotent; identical twins possible
Coelom arises by splitting of mesoderm	Coelom arises between invaginating mesoderm during gastrulation
Lophotrochozoans and Ecdysozoans	Echinoderms, Acorn worms, and Chordates

Let's first examine the protostomes. The deuterostomes are discussed below.

Lophotrochozoans vs. Ecdysozoans

Genome analysis, especially the analysis of 18S rRNA genes and HOX genes supports a major division of the **Protostomia** into two superphyla: **Lophotrochozoans** and **Ecdysozoans.**

Lophotrochozoans

Their name was created from the names of formerly-separated groups that have now been joined in a single clade on the basis of the similarities of their genomes. They all share a cluster of HOX genes quite different from those found in the ecdysozoans (and deuterostomes). They share similar sequences in their 18S rRNA genes. The clade contains a number of phyla of which we shall examine only 3.

- **flatworms** (Platyhelminthes),
- **annelids** (Annelida), and
- **mollusks** (Mollusca).

Flatworms (Phylum Platyhelminthes)

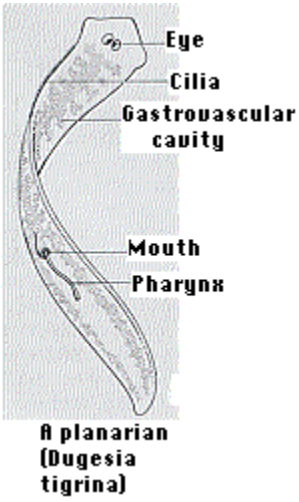


Figure 19.1.10.4 Flatworm

This phylum contains some 20,000 species distributed among three classes. **Turbellaria**, free-living forms of which the **planarian** is a commonly-studied example. Planaria share with the other members of the phylum (1) a flat, almost ribbonlike, shape and (2) bilateral symmetry. The bilateral symmetry of planarians is associated with active locomotion by secreting a layer of mucus underneath them and propelling themselves forward with the many cilia on their ventral surface and by swimming and a concentration of sense organs in the head (called **cephalization**). Planarians feed through a mouth on their ventral surface. It leads to an elaborate gastrovascular cavity. But there is no separate exit so undigested food has to leave by the mouth.

- **Trematoda**, a group of parasitic
  - lung flukes
  - liver flukes
  - blood flukes (e.g., **Schistosoma**)
  - All of these have at least two different stages in their life cycle, each parasitic in a different host - one of which is usually a snail.

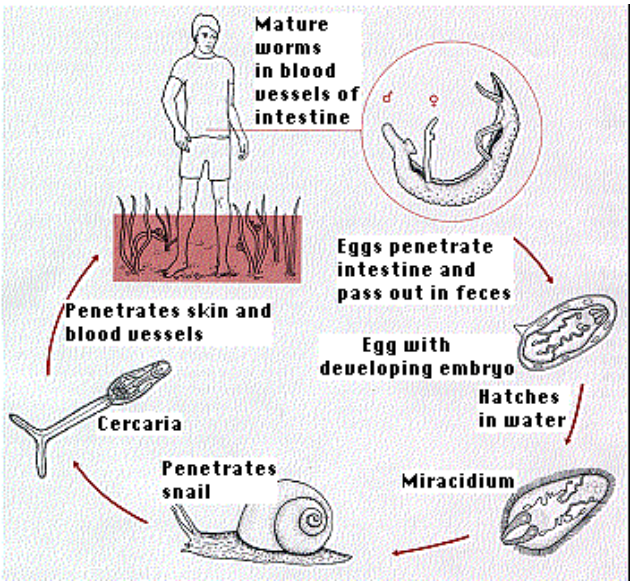


Figure 19.1.10.5 Lifecycle of Schistosoma

The diagram gives the life cycle of the blood fluke, ***Schistosoma mansoni***. Once within the alternate host, a snail, a single miracidium may produce as many as 200,000 infectious cercariae. Both sexes must infect the human if the cycle is to continue. With the increasing use of irrigation in tropical regions, the incidence of human infection — known as schistosomiasis or bilharzia — is rising alarmingly.

- **Cestoda**; the parasitic **tapeworms**. They, too, alternate between an intermediate host (e.g., pig, fish) and a definitive host (e.g., us). The growing popularity of sushi and sashimi made of raw Pacific salmon has caused infections by the fish tapeworm to become more common in the U.S.

Annelids (Phylum Annelida)

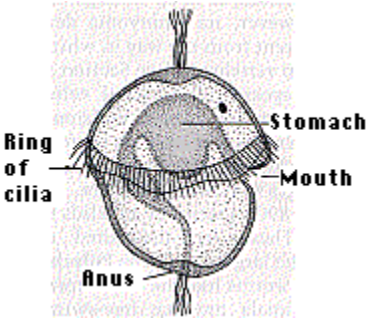


Figure 19.1.10.6 Trocophore larva

Characteristics:

- **segmented**; that is, their body is made up of repeating units. Although some structures, e.g., the digestive tract, run straight through, others like the excretory organs are repeated in each segment.
- The major nerve trunk runs along the **ventral** side.
- a large, fluid-filled **coelom**; It is lined with mesoderm and enables the internal organs to slide easily against one another making for easy locomotion.

There are >15,000 species known. Some examples:

- the common earthworm
- leeches
- marine forms such as the clam worm These animals produce a free-swimming **trochophore larva** (figure), which partly accounts for the name Lopho**troch**ozoan.

Mollusks (Phylum Mollusca)

With over 100,000 living species identified so far, the mollusks must be counted as among the most successful animals on earth today. Most belong to the first 3 of the 6 classes shown here:

1. **Bivalvia**. Two shells encase the body. Includes the clams, mussels, oysters, and scallops.
2. **Gastropoda**. Snails and slugs. Snails have a single shell ("univalves') while slugs have none.
3. **Cephalopoda**. This marine group includes the various species of octopus, squid, as well as the chambered nautilus. A record 28-foot (8.5 m) octopus and 60-foot (18 m) squid make these the largest of all the invertebrates.
4. **Scaphopoda**. Marine, filter-feeding "tooth shells".
5. **Monoplacophora**. Until a live specimen was discovered in 1952, these animals were thought to have been extinct for millions of years. It has a single shell (hence the name) and, unlike the other mollusks, is segmented (as are its relatives the annelids).
6. **Polyplacophora**. The animals in this group, called **chitons**, have their dorsal surface protected by 8 overlapping plates or "valves".

The trochophore larvae of mollusks is also evidence that they belong in the same clade with the annelids.

Ecdysozoans

All the members of this **clade**

- grow by periodically **molting -** shedding their skin or exoskeleton
- share a unique pattern of HOX genes, e.g. **Ubx** and **Abd-B**

The clade includes a number of phyla of which we shall examine 2:

- **nematodes**
- **arthropods**.

Roundworms (Phylum Nematoda)

Features:

- A one-way digestive tract running from mouth to anus.
- A cavity between the digestive tract and the body wall. It develops from the blastocoel and is called a **pseudocoel**.
- Some 25,000 species have been identified but this may be less than 10% of the true number.
- Most are free-living; found in soil where they are important decomposers.
- One of these is ***Caenorhabditis elegans***, a model laboratory animal.
- Some are parasitic, including
  - hookworms (In 2003 the number of humans infected by hookworms was estimated at 740 million worldwide.)
  - pinworms and whipworms
  - filarial worms - threadlike worms that are transmitted to the definitive host from an intermediate host causing such human ailments as
    - river blindness (*Onchocerca volvulus*) - acquired from the bite of infected black flies
    - elephantiasis (*Wuchereria bancrofti*) - acquired from infected mosquitoes
    - dracunculiasis (Guinea worm disease) (*Dracunculus medinensis*) - acquired from ingesting water containing infected "water fleas" (Cyclops)
  - many parasites of commercially important plants like strawberries and oranges.
- Most are small although one that parasitizes whales reached 30 feet (9 m)!

Arthropods (Phylum Arthropoda)

Some characteristics:

- Incredible diversity. Over a million living species have been identified so far - more than all the other species of living things put together - and this is probably only a fraction of them.
- Live in every possible habitat: fresh water, salt water, soil, even in the most forbidding regions of Antarctica and high mountains.
- A jointed external skeleton made of **chitin**, a polymer of N-acetylglucosamine (NAG).
- Segmented.
- Pairs of jointed appendages; one pair to a segment - used for locomotion, feeding, sensation, weaponry.
- Bilateral symmetry.
- Main nerve cord runs along the ventral side.

We shall look at four groups (subphyla):

- **Crustacea**
- **Hexapoda** (the insects)
- **Myriapoda**
- **Chelicerata**

Crustacea

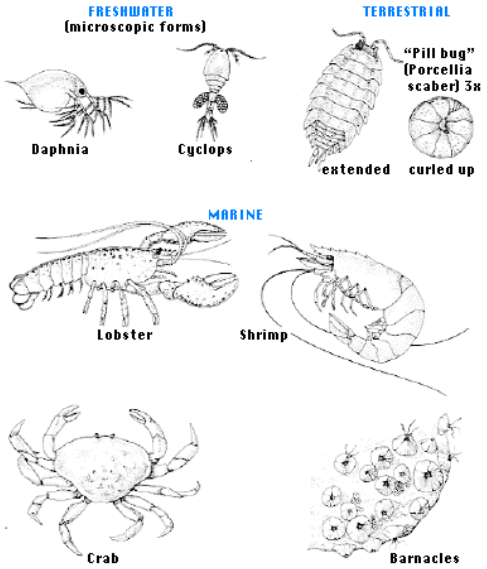


Figure 19.1.10.7 Crustacea

- Head and thorax fused into a **cephalothorax**.
- At least 40,000 species.
- Most are aquatic, found in both fresh water and in the oceans.
- Includes crayfish, lobsters, barnacles, crabs, shrimp.

Hexapoda - the insects

- Body segments grouped into **head**, **thorax**, and **abdomen**.
- Each of the 3 thoracic segments carries a pair of legs (hence the **6-legged** "hexapoda")
- Many have wings, usually 2 pairs (only one pair in flies - diptera).
- Gas exchange through a tracheal system.
- Nitrogenous waste is uric acid thus conserving water.
- Some 950,000 species, and this may be only 10% of the number out there.
- Dominate all habitats except for the oceans.
- Most intensively-studied representative: ***Drosophila melanogaster***.
- Representative colonial insect: the honeybee, ***Apis mellifera***

Myriapoda

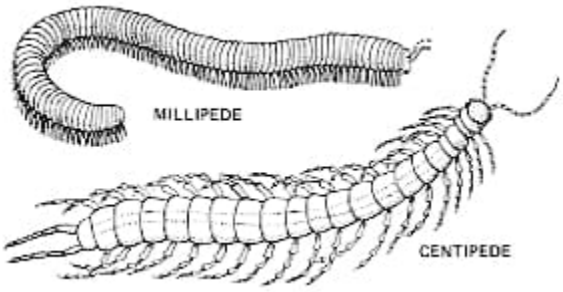


Figure 19.1.10.8 Millipede

Some 13,000 species of

- **centipedes**
- **millipedes**

Neither group has the number of legs their name suggests, although one species of millipede does have 375 pairs.

Chelicerata

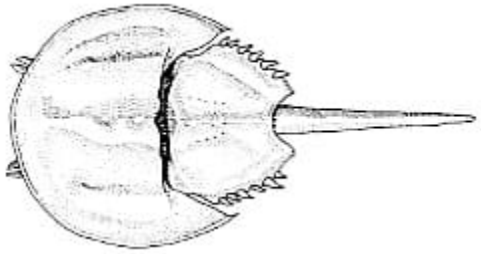


Figure 19.1.10.9 Limulus

- Anterior segments fused into a **cephalothorax**.
- The first pair of appendages - the chelicerae - are used for feeding.
- There are no antennae.
- Includes:
  - **Merostomata**. The only member alive today is **Limulus**, the horseshoe "crab". It has existed in the sea virtually unchanged for 200 million years.
  - **Arachnids** (some 75,000 species)
    - 8-legged
    - scorpions, mites, ticks, spiders, daddy longlegs.

Evolutionary relationships of the arthropods

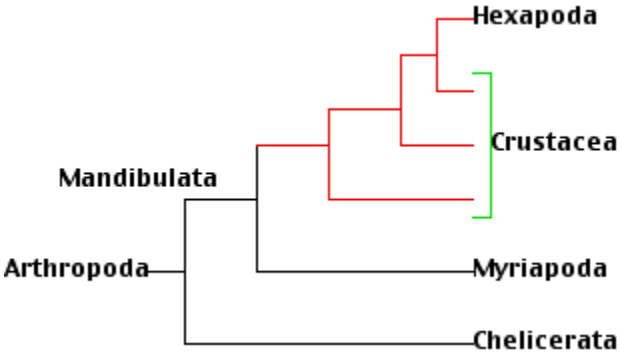


Figure 19.1.10.10 Anthropod sequence

An ever-increasing number of arthropod gene sequences appear to have answered some long-standing questions about the evolutionary relationships of the various arthropod groups. A recent study (Regier, J. C., *et al.*, **Nature**, **463**:1079, 25 February 2010) examined 63 nuclear genes from 75 species of arthropods and concluded that

- the crustacea are paraphyletic; that is, the single common ancestor from which all the animals we call crustaceans are descended was also the ancestor of another group, the insects (**Hexapoda**). So insects are terrestrial crustaceans!
- All these groups plus the millipedes and centipedes (**Myriapoda**) make up a clade designated **Mandibulata**.
- So millipedes and centipedes are more closely related to the crustaceans than to, as once thought, the **Chelicerata**.

The Deuterostomes

In addition to the features listed above, the deuterostomes have (or had) **gill slits**. (The echinoderms have lost the gill slits of their ancestors.)

Echinoderms (Phylum Echinodermata)

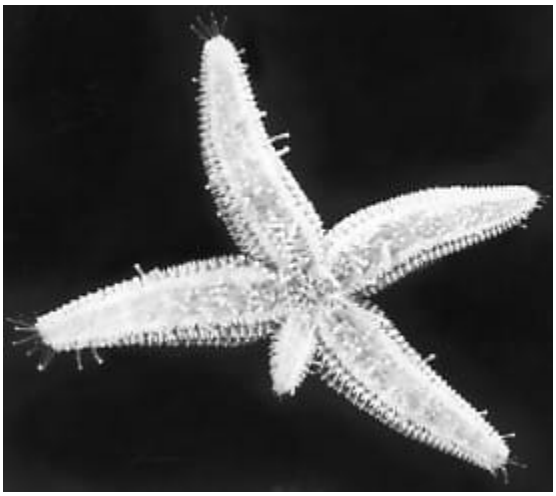


Figure 19.1.10.11 Starfish

Characteristics:

- **radial symmetry**. HOWEVER, their larvae have **bilateral symmetry** so the echinoderms probably evolved from bilaterally symmetrical ancestors and properly belong in the Bilateria.
- **water vascular system**. Seawater is taken into a system of canals and is used to extend the many **tube feet**. These have suckers on their tips and aid the animal in attaching itself to solid surfaces.
- no gill slits
- About 6,000 species — all of them marine.

There are 5 classes of echinoderms:

- Sea lilies (**Crinoidea**)
- Sea Stars (aka "Starfish") (**Asteroidea**) The photo (courtesy of Dr. Charles Walcott) shows a sea star that lost an arm and is in the process of regenerating a replacement.
- Brittle stars (**Ophiuroidea**)
- Sea cucumbers (**Holothuroidea**)
- Sea urchins and sand dollars (**Echinoidea**)

Acorn Worms (Phylum Hemichordata)

The members of this small phylum (some 90 species have been identified) are marine forms most of whom live in burrows in ocean sediments. Their closest living relatives are the **echinoderms** with which they share the clade **Ambulacraria**. However, they possess a suite of features, both in their anatomy (e.g. gill slits) and their gene expression patterns, suggesting that their ancestors also led to the evolution of the chordates.

Chordates (Phylum Chordata)

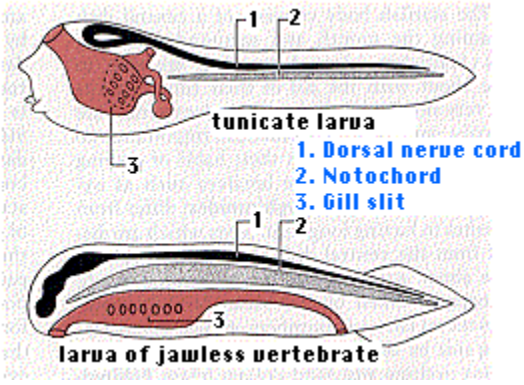


Figure 19.1.10.12 Larva of jawless vertebrate

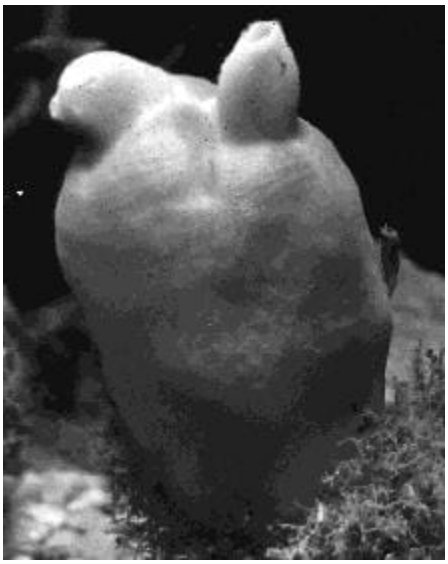
During their embryonic development, all chordates pass through a stage called the **pharyngula** with these features:

- a **dorsal, tubular nerve cord** ("**1**") running from anterior to posterior. At its anterior end, it becomes enlarged to form the **brain**.
- a flexible, rodlike **notochord** ("**2**") that runs dorsal to the digestive tract and provides internal support. In vertebrate chordates, it is replaced by a vertebral column or backbone long before maturity.
- pairs of **gill pouches**. These lateral outpocketings of the pharynx are matched on the exterior by paired grooves. In aquatic chordates, one or more pairs of gill pouches break through to the exterior grooves, forming **gill slits** ("**3**"). These provide an exit for water taken in through the mouth and passed over the gills.
- a tail that extends behind the anus
- The vast majority of chordates have a skull enclosing their brain (**Craniata**), and all but one of these (the hagfish) convert their notochord into a vertebral column or **backbone**. These latter are the **vertebrates**.

  Vertebrates also differ from all the other animals by having quadrupled their HOX gene cluster; that is, vertebrates have 4 clusters of HOX genes located on 4 different chromosomes.

  Here we shall examine two groups of **invertebrate chordates**:
  - **Urochordata** and
  - **Cephalochordata**

Urochordata


Figure 19.1.10.13 Tunicata

This group (also called **Tunicata**) includes animals known as **ascidians** (and commonly called **sea squirts**). They are

- marine
- sessile animals
- feed by filtering food particles from seawater taken in through one opening, or siphon, and squirted out the other.

The one on the above is ***Halocynthia***, the sea peach (photo courtesy of Ralph Buchsbaum). It is hard to see what makes these animals chordates. The adults have neither notochord nor a dorsal tubular nervous system. However, these animals disperse themselves with free-swimming larvae that have

- **dorsal tubular nervous system**
- **notochord**
- **gill slits**

One of the most common species (***Ciona intestinalis***) has had its genome sequenced.

- It has a very small genome: ~1.6 x 10^8 base pairs encoding ~16,000 genes. (Some 20% of these are organized in operons.)
- Its larva is small (with ~2,600 cells) including only
  - 36 muscle cells
  - 40 notochord cells
  - 100 neurons
- These cells (as well as the others) develop along rigid pathways which can be easily observed because the larva is
- transparent.

All these features are shared with *C. elegans*, but now we are talking about an animal far closer to the evolutionary line that produced us. In fact, with 80% of Ciona's genes having homologs in us, tunicates are probably our closest invertebrate relatives.

Cephalochordata

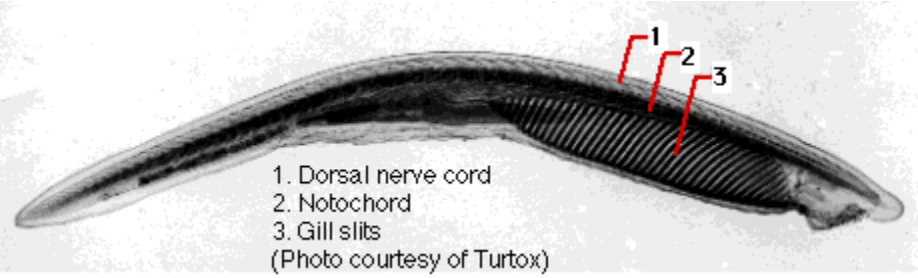


Figure 19.1.10.14 Amphioxus

The representative member of this tiny subphylum of so-called lancelets is a small (5 cm), marine, fishlike creature called amphioxus (above). For years its genus name was **Amphioxus** but that has now been replaced by the name Branchiostoma. Amphioxus retains a **dorsal nerve cord, notochord** and **gill slits** throughout its life. There is a small cluster of neurons at the anterior tip of the nerve cord with certain similarities of structure and gene expression to the vertebrate fore-, mid- and hindbrain. Although able to swim, the lancelet spends most of its time partially buried in the sand while it filters microscopic food particles from the water.

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19.1.11: Drosophila Melanogaster

This page outlines the advantages of using Drosophila (fruit flies) in genetic studies, highlighting their small size, rapid life cycle, and high reproduction rates which support statistically significant results. It mentions the presence of giant polytene chromosomes in larvae for structural analysis, the external development of embryos for easy observation, and the small genome that allows for targeted mutations, all contributing to their effectiveness in research.

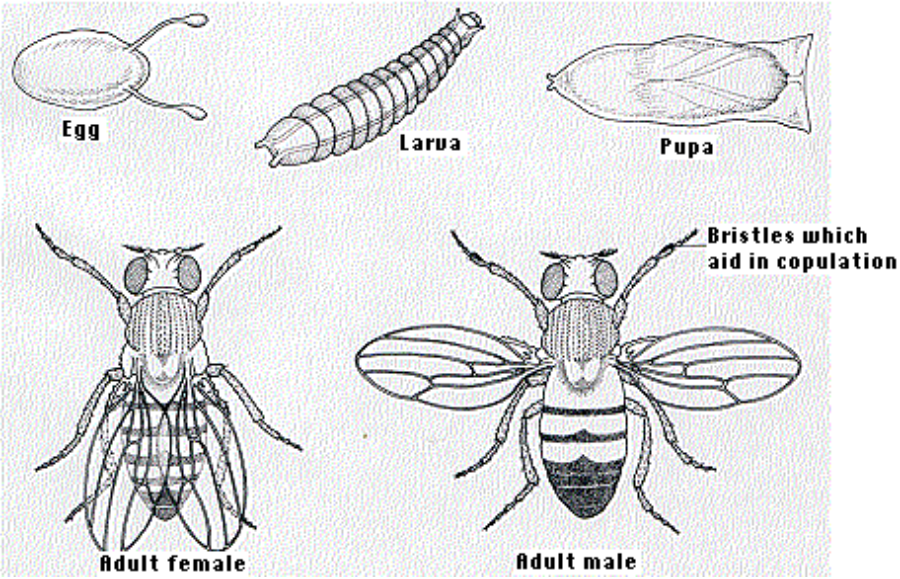


Figure 19.1.11.1 Stages of Drosophila

Some of the reasons for its popularity:

- The flies are small and easily reared in the laboratory.
- They have a short life cycle The figure shows the various stages of the life cycle (not all drawn to the same scale). A new generation of adult flies can be produced every two weeks.
- They are fecund; a female may lay hundreds of fertilized eggs during her brief life span. The resulting large populations make statistical analysis easy and reliable.
- The giant ("polytene") chromosomes in the salivary (and other) glands of the mature larvae.
  - These chromosomes show far more structural detail than do normal chromosomes
  - They are present during interphase when chromosomes are normally invisible.
- More recently, Drosophila has proven in other ways to have been a happy choice.
  - Its embryo grows outside the body and can easily be studied at every stage of development.
  - The blastoderm stage of the embryo is a syncytium (thousands of nuclei unconfined by cells) so that, for example, macromolecules like DNA injected into the embryo have easy access to all the nuclei.
  - The genome is relatively small for an animal (less than a tenth that of humans and mice).
  - Mutations can targeted to specific genes.

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19.1.12: Caenorhabditis Elegans

Caenorhabditis elegans is a microscopic (~1 mm) nematode (roundworm) that normally lives in soil. It has become one of the "model" organisms in biology. It is a true animal with at least rudiments of the physiological systems - feeding, nervous, muscle, reproductive - found in "higher" animals like mice and humans. However, it is so small that large numbers can be raised in petri dishes (where it is fed *E. coli* - another model organism). It reproduces rapidly.

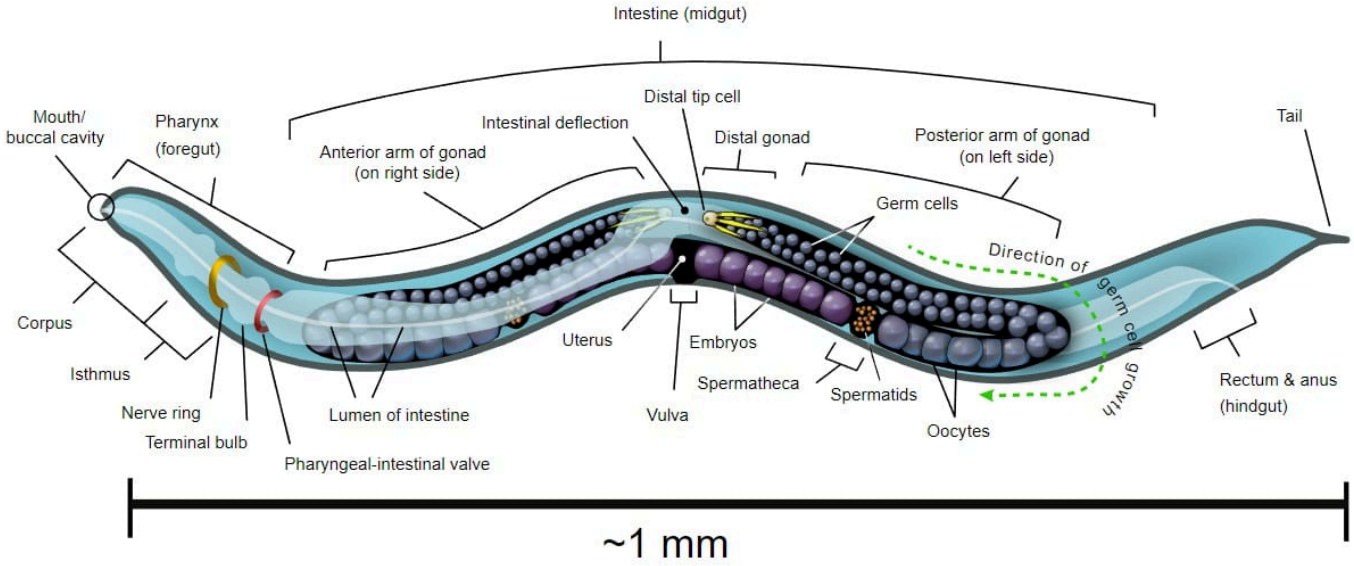


Figure 1: A lateral (left side) anatomical diagram of an adult-stage nematode hermaphrodite Caenorhabditis elegans (C. elegans) with emphasis on the digestive and reproductive systems. (CC BY-SA 3.0; K. D. Schroeder).

It is transparent so that every cell in the living animal can be seen under the microscope from the fertilized egg to the 556 cells of the newly-hatched worm and, later, the 959 somatic cells, and a variable number of germ cells, of the adult worm. It can be easily transformed with transgenes - DNA injected into the animal. It can also be treated with antisense RNA. Before it dies (after 2–3 weeks), it shows signs of aging and thus may provide general clues as to the aging process.

Its cells contain 5 pairs of autosomes and 2 X chromosomes. These animals are hermaphrodites, producing both sperm and eggs. Most of the time they fertilize themselves, so that any recessive alleles quickly become homozygous and affect the phenotype. On rare occasions, nondisjunction occurs during meiosis with the loss of one X chromosome. Animals with a single X are males and are able to fertilize the eggs of the hermaphrodites (with more success than they have themselves).

C. elegans was the first multicellular eukaryote to have its entire genome sequenced. It contains some 19–20,000 protein-encoding genes incorporated in 100,258,171 base pairs of DNA. In contrast to other eukaryotes, some 13–15% of its genes are grouped in **operons** containing 2–8 genes each.

C. elegans Fertilization

Like all animals, *C. elegans* starts life as a fertilized egg (zygote) which then undergoes the mitotic divisions needed to produce the adult. Because the worm is transparent and the pattern of differentiation is so rigid it has been possible to trace the lineage of every single somatic cell in the animal. Just after hatching, the it contains 556 cells and is approximately 0.3 mm long. After reaching maturity, it will contain 959 somatic cells and a variable number of germ cells in its gonad.

The diagrams below show the pathway by which each of the 556 cells in the larva of *C. elegans* has developed from the zygote. The relative length of the vertical lines indicates the length of the interval before the next mitosis. Some pathways end in the programmed death of the cell (apoptosis) even before the larva is complete.

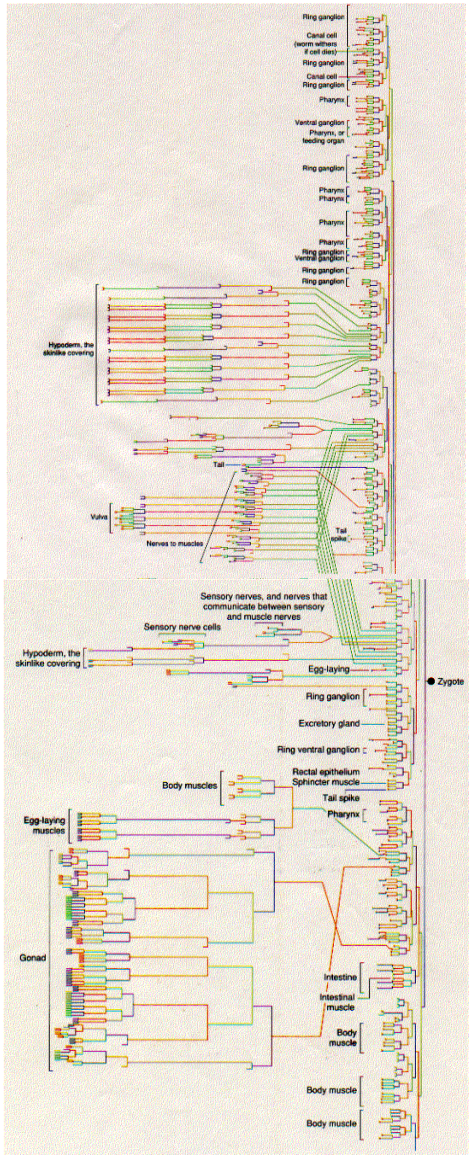


Figure 19.1.12.2 Pathway of 556 cells in larva of C.elegans. (Adapted, with permission, from J. Sulston, et al., in Monograph 17. Copyright © Cold Spring Harbor Laboratory, NY.)

Several remarkable features have been found from these studies. The pattern of development is invariable from worm to worm. Every one of the 556 cells that make up the newly-hatched larva develops from a rigid pattern of mitotic division leading back to the zygote. 131 cells in the developing embryo die by apoptosis. This cell death is not random; which cells will die and at what stage is completely predictable. Any failures in this programmed cell death can lead to serious abnormalities.

Each organ - skin, nerve, muscle, etc. - is made of cells derived from several different lineages. One might have expected that the earliest cell divisions would produce daughter cells destined to go on to form a single structure in the embryo. But that is not generally the case. Instead, most of the earliest cells will produce descendants that team up with other groups of cells to form the various organs of the animal.

There is less flexibility of cell fate than occurs in mammals (or even amphibians). With a microscopic laser beam, a single cell can be killed in the developing embryo. Often the result is that all the cells that would have descended from that cell fail to form. Neighboring cells may not compensate for the loss as they do so freely in mammals. So it appears that the developmental controls of *C. elegans* rely more on **cell-intrinsic signals** rather than inductive signals. But this distinction is relative, not absolute. Cells originally destined to a different fate will in some cases switch their path of differentiation to replace the cells killed by the laser. In these cases, the switch is clearly mediated by **inductive signals** liberated by nearby cells.

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19.1.13: Vertebrates

This page outlines the evolutionary stages of chordates, emphasizing the pharyngula stage in embryos and the classification of craniates into vertebrates and invertebrates. It details the characteristics and adaptations of jawless and jawed fishes, amphibians, and the evolution of amniotes.

Chordata

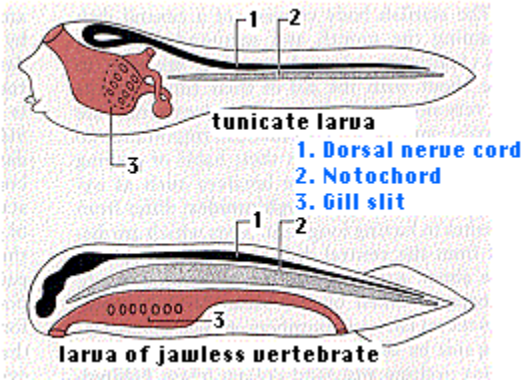


Figure 19.1.13.1Turnicate larva

During their embryonic development, all chordates pass through a stage called the **pharyngula** with these features:

- The cephalochordates and tunicates never develop a vertebral column. They are thus "invertebrates" and are discussed with the other invertebrates.
- Craniata The vast majority of chordates have a skull enclosing their brain, eyes, inner ear, etc.). All but one group of these (the hagfishes) also convert their notochord into a vertebral column or **backbone** thus qualifying as **vertebrates**.

Vertebrata

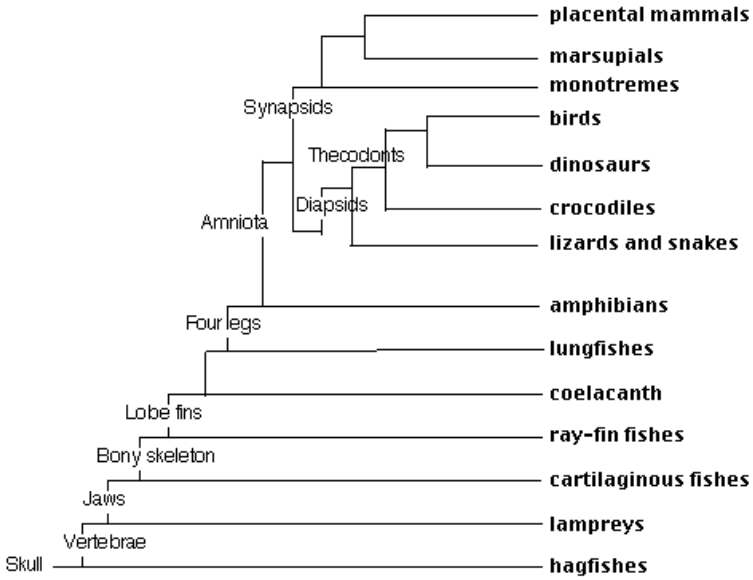


Figure 19.1.13.2 Vertebrate clade

Although **hagfishes**, never replace their notochord with a vertebral column, and thus might seem not to qualify as vertebrates, they share a number of other features with other vertebrates and certainly should be classified with them. Still uncertain is whether they represent the most primitive vertebrates or are simply degenerate vertebrates (probably the latter).

All the other members of the craniata convert their notochord into a **vertebral column** or "backbone" (even though in some it is made of cartilage not bone). They also differ from all other animals in having quadrupled their HOX gene cluster; that is, they have 4 different clusters of HOX genes (on 4 separate chromosomes). Perhaps this acquisition played a key role in the evolutionary diversity that so characterizes the vertebrates.

The vertebrates are subdivided into the

- jawless vertebrates (**Agnatha**)
- jawed vertebrates (**Gnathostomata**)

Agnatha



Figure 19.1.13.3 Lamprey

Lampreys and hagfishes are the only jawless vertebrates to survive today. They both have a round mouth and for this reason are often referred to as **cyclostomes**. They are the most primitive of the vertebrates. By "primitive", a biologist means that they are the least changed from the first vertebrates. Besides lacking jaws,

- They have no paired pectoral (shoulder) or pelvic (hip) fins.
- Their notochord persists for life, never being completely replaced by a backbone even in the lampreys.
- They have no scales.
- The axons of their neurons are unmyelinated (like those of all invertebrates).
- Lampreys have both an innate immune system and an adaptive immune system, but the latter is entirely different from that found in the jawed vertebrates.

The photo (courtesy of the Carolina Biological Supply Company) is of the West Coast lamprey. Note the gill slits and the absence of paired pectoral and pelvic fins.

Gnathostomata

As well as having jaws, all the members of this group have

- Myelin sheaths around the axons of their neurons. This permits much more rapid transmission of nerve impulses - a trait probably as important for active vertebrates as their jaws.
- An adaptive immune system backing up their innate immune system.

Cartilaginous Fishes (Chondrichthyes)

Fossils of cartilaginous fishes become abundant in deposits dating to the Devonian period. They were very much like the sharks of today. The group, which today is made up of some 1,188 species of **sharks, skates** and **rays** gets its name from the fact that their skeleton is made of cartilage, not bone.

With their gills exposed to sea water, all marine fishes are faced with the problem of conserving body water in a strongly hypertonic environment. Sea water is about 3.5% salt, over 3 times that of vertebrate blood. The cartilaginous fishes solve the problem by maintaining such a high concentration of **urea** in their blood (2.5% — far higher than the ~0.02% of other vertebrates) that it is in osmotic balance with - that is, is isotonic to - sea water.

This ability develops late in embryology, so the eggs of these species cannot simply be released in the sea. Two solutions are used:

- Enclose the egg in an impervious case filled with isotonic fluid before depositing it in the sea.
- Retain the eggs and embryos within the mother's body until they are capable of coping with the marine environment.

Both these solutions require **internal fertilization**, and the cartilaginous fishes were the first vertebrates to develop this. The pelvic fins of the male are modified for depositing sperm in the reproductive tract of the female.

Bony Fishes (Osteichthyes)

As their name indicates, the skeleton in this group is made of bone. The group is subdivided into the

- ray-finned fishes (**Actinopterygii**)
- **lobe-finned fishes** (**Sarcopterygii**)

Ray-finned fishes

- Their fins are thin and supported by spines.
- There are over 30,000 species (representing more than half of all living vertebrates).
- They are an important part of the human diet in many areas of the world and, in affluent nations, support a large sports fishing industry.

Although the earliest bony fishes may have appeared late in the Silurian period, their fossils become abundant in freshwater deposits of the Devonian period. In addition to gills, these fishes had a pair of pouched outgrowths from the pharynx which served as **lungs**. They were inflated with air taken in through the mouth and may have provided a backup gas exchange organ when the water became too warm and stagnant to carry enough dissolved oxygen. Their kidneys were adapted for the hypotonic environment in which they lived.

These animals diversified through the remainder of the Devonian period (which is often called the **"Age of Fishes"**). Some migrated to the oceans. In this more stable environment, their lungs became transformed into a **swim bladder** with which they could alter buoyancy. Their kidneys became transformed as well adapting them to their new - hypertonic - surroundings.

Lobe-finned fishes

The only ones to survive today are:

- two species of **coelacanths**. Coelacanths were long thought to have become extinct at the end of the Mesozoic era, some 70 million years ago. But in December 1938, a living coelacanth, **Latimeria chalumnae**, was pulled up from the depths of the ocean off the east coast of Africa. Since then, over 200 additional specimens have been caught.
- several species of **lungfish** found in Africa, South America, and Australia.

The nostrils of bony fishes open only to the outside and are used for smelling. Some of the lobe-finned fishes developed **internal openings to their nostrils**. This made it possible to breath air with the mouth closed as modern lungfishes do.

Judging from present-day lungfishes, two other significant adaptations evolved in this group:

- two atria and a partial septum in the ventricle of the heart (similar to the frog heart). This permitted a partial separation of oxygenated blood returning from the lung(s) and the deoxygenated blood returning from the rest of the body.
- an enzyme system to convert **ammonia** into the less toxic **urea**. This mechanism is highly-developed in the African and South American lungfishes. While in the water, these fishes excrete their waste nitrogen as ammonia, just as most ray-finned fishes do. In time of drought, these animals burrow in the mud and switch to urea production.

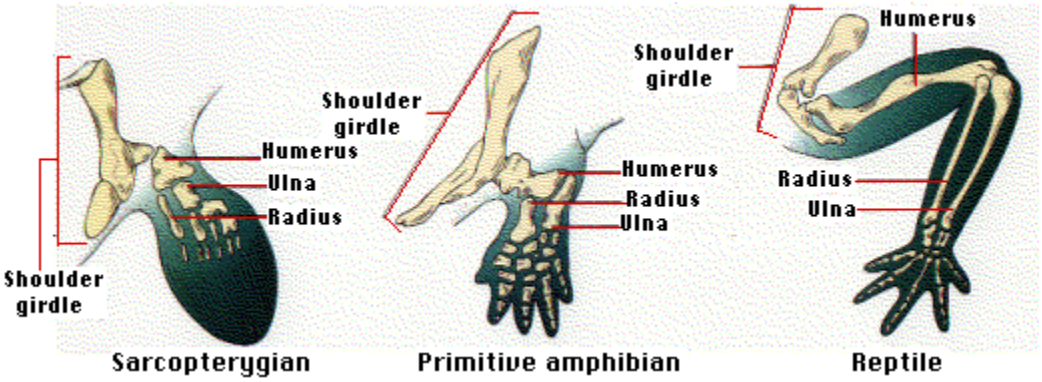


Figure 19.1.13.5 Frog

With their bony limbs and lungs inherited from their lobe-finned ancestors, amphibians were so successful during the Carboniferous (Mississippian and Pennsylvanian periods) that these periods are known as the **Age of Amphibians**.

The Carboniferous was followed by the Permian, when the earth became colder and dryer. The fortunes of the amphibians began to decline until only three groups - totaling about 6500 species - remain today:

- frogs and toads (**Anura**) (The one pictured is ***Rana pipiens***, the leopard frog.)
- salamanders and newts (**Urodela**)
- caecilians (**Apoda**), which are rare, limbless, tropical animals.

As the name suggests, amphibians are only semiterrestrial:

- Their skin is soft and moist so they are at risk of desiccation in dry surroundings.
- Their eggs have no waterproof covering so
  - they must be laid in water (which makes them useful animals for studying embryonic development) where they are fertilized or
  - placed within the mother's body (some use a pouch in the skin, some use their mouth, some even use their stomach — which stops secreting acid and enzymes for the duration!) after **external fertilization**.

Amniotes (Amniota)

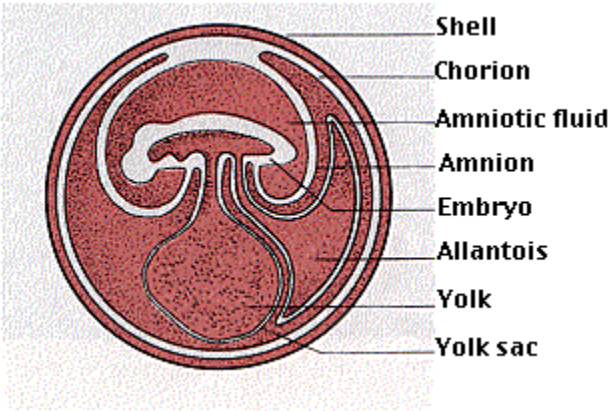


Figure 19.1.13.6 Amniotic egg

Some 310 million years ago (in the Pennsylvanian), some amphibians evolved the ability to lay shelled, yolk-filled eggs. The

embryo developing within the egg produces **4 extraembryonic membranes**:

- amnion, which surrounds the embryo with a fluid as watery as the pond water around a frog's egg (and accounts for the name amniota)
- **chorion**, which serves for gas exchange
- **allantois**, which serves both for gas exchange and to store metabolic wastes
- **yolk sac**, which supplies the embryo with food



Figure 19.1.13.7 Chameleon

With the arrival of the cold, dry Permian, reptiles were well-adapted to survive because of their development of a **shelled, yolk-filled egg** which could be deposited on land without danger of drying out. The photo (courtesy of the Carolina Biological Supply Company) shows an American chameleon emerging from its egg.

Other adaptations that enabled the reptiles to flourish for the next 220 million years were:

- a dry, water-impermeable skin
- lungs inflated by expansion of the rib cage
- a partial septum in the ventricle reducing the mixing of oxygenated and deoxygenated blood

Beginning late in the Paleozoic era and exploding in the Triassic period, the reptiles underwent a remarkable adaptive radiation producing the **diapsids**. This group developed the ability to convert their nitrogenous waste into **uric acid**. Uric acid is almost insoluble in water so its excretion involves little loss of water. (It is the whitish paste that pigeons leave on statues.) This modification largely freed the diapsids and their descendants from a dependence on drinking water; the water in their food is usually sufficient.

Diapsid evolution soon produced:

- **lizards** and **snakes** (Squamata - some 6,300 species survive today);
- **turtles**
- **thecodonts**.

Thecodonts were able to run fast by rising up on their hind legs, which became larger than their front legs, and using their long tail for balance. The group diversified into:

- **crocodiles** and **alligators** (Crocodilia — 22 species survive today)
- an extraordinary array of **dinosaurs** from some of which evolved today's **birds**.

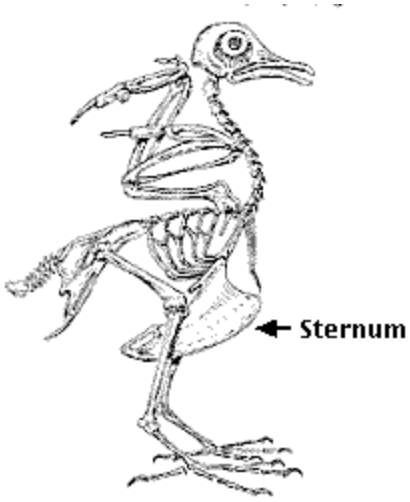


Figure 19.1.13.8 Pigeon sternum

Feathers are the feature that most clearly distinguishes the birds from their dinosaur ancestors. These scaly skin outgrowths provide a light, strong surface for the **wings**;

- heat insulation, making it possible to be small but still warm-blooded.

Other adaptations are:All of these adaptations help birds to fly (to escape predators and find suitable food and nesting sites). Almost 10,000 species are known today.

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19.1.14: Zebrafish

The zebrafish, *Danio rerio*, has become another popular "model" organism with which to study fundamental biological questions.

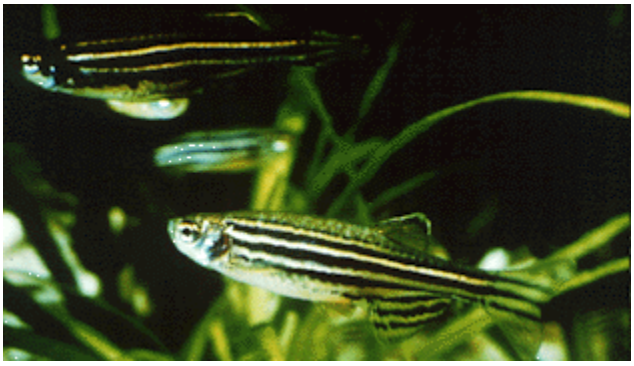


Figure 19.1.14.1 Zebrafish This image is courtesy of Sarah Farrington of the Center for Cancer Research at MIT. It comes from the home page of ZFIN ("Zebrafish Information Network").

The zebrfish is a small (1–1.5 inches)(2.5–3.8 cm) freshwater fish that grows easily in aquaria (it is available at many pet stores). Some of its advantages for biologists:

- It breeds early and often (daily).
- It is a vertebrate, like us, and thus can provide clues to human biology that invertebrates like *Drosophila* and *Caenorhabditis elegans* may not.
- Its embryos, like those of most fishes, develop outside the body where they can be easily observed (unlike mice).
- Its embryos are transparent so defects in development can be seen easily.
- Individual cells in the embryo can be labeled with a fluorescent dye and their fate followed.
- Embryonic development is quick (they hatch in two days).
- They can absorb small molecules, such as mutagens, from the aquarium water.
- Individual cells - or clusters of cells - can be transplanted to other locations in the embryo (as Mangold did with newt embryos).
- They can be forced to develop by parthenogenesis to produce at will homozygous animals with either a male-derived or female-derived genome.
- They can be cloned from somatic cells.
- They can be made transgenic (like mice and *Drosophila*)
- Its genome (1.4×10^9 base pairs) has been sequenced revealing 26,606 protein-coding genes.

Forward and Reverse Genetics

Forward

Since Mendel's time, most genetics has involved

- observing an interesting **phenotype**
- tracking down the gene responsible for it.

So this "forward" genetics proceeds from phenotype -> genotype.

Some examples:

- Mendel's work
- RFLP analysis of large families
- The one gene - one enzyme theory

These methods have been called "forward" genetics to distinguish them from a more recent approach, which has become an urgent priority with the successes of genome sequencing.

Reverse

Rapid methods of DNA sequencing has generated a vast amount of data. Thousands of suspected genes have been revealed (e.g., finding open reading frames - ORFs), but the function of many of them is still unknown.

But now with a knowledge of the DNA sequence of a gene of unknown function, one can use methods for suppressing that particular gene ("knockdown") and then observe the effect on the phenotype.

So this "reverse" genetics proceeds from genotype -> phenotype.

Reverse genetics has been applied successfully to

- plants
- mice
- *C. elegans*
- zebrafish

For example, the function of a mysterious gene sequence in *Danio* can be studied by

- synthesizing a short **antisense** oligonucleotide complementary to a section of the gene.
- The oligonucleotide is chemically-modified to make it more stable than a fragment of RNA.
- Binding to its complementary sequence on the messenger RNA (mRNA) produced by transcription of the animal's gene, blocks ("knocks down") gene expression by
  - preventing translation or
  - disrupting normal splicing of the mRNA.

Because we share so many similar gene sequences (orthologous genes) with *Danio*, if one can discover the function of the gene in *Danio*, then we have a better idea of the role of its ortholog in humans.

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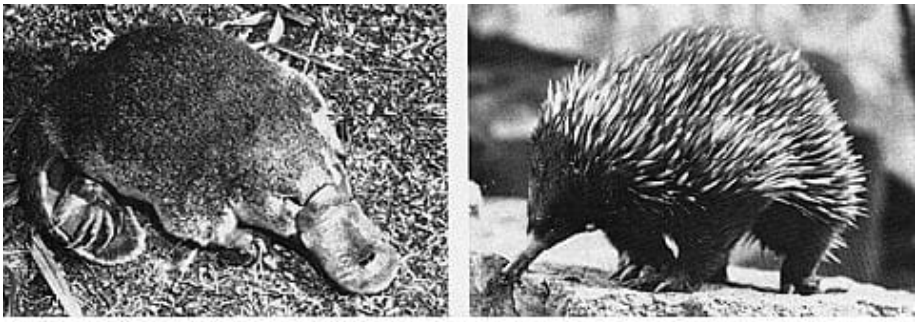
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19.1.15: Monotremes

This page discusses monotremes, a unique group of mammals including the duckbill platypus and spiny anteaters, which exhibit both mammalian and reptilian traits. They have a cloaca, lay shelled eggs, and nurse with milk lacking nipples. Recent genomic research has revealed the platypus possesses 18,527 protein-coding genes, showcasing a mix of characteristics from mammals and reptiles, including genes for egg yolk proteins, numerous underwater hunting receptors, and unique venom-producing genes.

Monotremes are a small but remarkable group of mammals that consists of a single species of **duckbill platypus** (*Ornithorhynchus anatinus*) found in Australia and three (perhaps four) species of spiny anteaters (**echidnas**) found in Australia and New Guinea.



Monotremes: mammals that lay eggs. The duckbill platypus (left) raises its young in a nest; the spiny anteater (echidna, on the right) places them in a pouch on its abdomen. (Courtesy of the Australian Information Service.)

These animals retain several traits of their therapsid ancestors including

- a cloaca - the final segment of the digestive tract into which both the urinary and reproductive tracts empty (monotreme = single hole);
- lay shelled eggs that undergo merobastic cleavage like that of reptiles (and birds) rather than the holoblastic cleavage of all other mammals.

Despite these reptilian features, the monotremes meet all the criteria of true mammals:

- milk secreted from **mammary glands** (but no nipples)
- **hair**
- **teeth** (only in the young; they are lost in adult montremes)

In the May 8 issue of **Nature**, a consortium of gene sequencers reported the results of sequencing the complete genome of the platypus. They identified 18,527 protein-coding genes distributed on 52 chromosomes.

The mix of mammalian and reptilian phenotypic features turns out to be reflected in the genome as well. Examples:

- The platypus has genes for egg yolk proteins that are also found in birds but not in therians.
- The gene content of their X chromosomes resembles that of the Z chromosome in birds, not the X chromosome of other mammals like us.

Other features of their genome reflect their unique biology:

- The platypus produces a venom with genes which in other mammals encode for antimicrobial peptides called defensins.
- The platypus has some 1000 genes for receptors in its vomeronasal organ — far more than found in other mammals. The platypus hunts for food underwater and probably uses these receptors to detect prey (as well as using its electroreceptors for this purpose).

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19.2: MICROBES



- Topic hierarchy

19.2A: Bacteria

19.2B: Archaea

19.2C: Antibiotics

19.2D: E. coli

19.2E: Anthrax

19.2F: Bacillus Thuringiensis

19.2G: The Rapid Identification of Microorganisms

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19.2A: Bacteria

Bacteria are microscopic organisms whose single cells have neither a membrane-enclosed nucleus nor other membrane-enclosed organelles like mitochondria and chloroplasts. Another group of microbes, the archaea, meet these criteria but are so different from the bacteria in other ways that they must have had a long, independent evolutionary history since close to the dawn of life. In fact, there is considerable evidence that you are more closely related to the archaea than they are to the bacteria!

Properties of Bacteria

- prokaryotic (no membrane-enclosed nucleus)
- no mitochondria or chloroplasts
- a single chromosome
  - a closed circle of double-stranded DNA
  - with no associated histones
- If flagella are present, they are made of a single filament of the protein **flagellin**; there are none of the "9+2" tubulin-containing microtubules of the eukaryotes.
- Ribosomes differ in their structure from those of eukaryotes
- Have a rigid cell wall made of **peptidoglycan**.
- The plasma membrane (in Gram-positive bacteria) and both membranes in Gram-negative bacteria are phospholipid bilayers but contain no cholesterol or other steroids.
- No mitosis
- Mostly asexual reproduction
- Any sexual reproduction very different from that of eukaryotes; no meiosis
- Many bacteria form a single **spore** when their food supply runs low. Most of the water is removed from the spore and metabolism ceases. Spores are so resistant to adverse conditions of dryness and temperature that they may remain viable even after 50 years of dormancy.

Classification of Bacteria

Until recently classification has done on the basis of such traits as:

- shape
  - bacilli: rod-shaped
  - **cocci**: spherical
  - **spirilla**: curved walls
- ability to form spores
- method of energy production (glycolysis for anaerobes, cellular respiration for aerobes)
- nutritional requirements
- reaction to the Gram stain

Figure 19.2.1.1 Gram positive and negative bacteria

Gram-positive bacteria are encased in a plasma membrane covered with a thick wall of peptidoglycan. Gram-negative bacteria are encased in a triple-layer. The outermost layer contains lipopolysaccharide (LPS).

- The bacterial cells are first stained with a purple dye called crystal violet.
- Then the preparation is treated with alcohol or acetone.
- This washes the stain out of **Gram-negative** cells.
- To see them now requires the use of a counterstain of a different color (e.g., the pink of safranin).
- Bacteria that are not decolorized by the alcohol/acetone wash are **Gram-positive**.

Although the Gram stain might seem an arbitrary criterion to use in bacterial taxonomy, it does, in fact, distinguish between two fundamentally different kinds of bacterial cell walls and reflects a natural division among the bacteria. More recently, genome

sequencing, especially of their 16S ribosomal RNA (rRNA), has provided additional insights into the evolutionary relationships among the bacteria.

Gram-positive Bacteria

Firmicutes

Comparison of their sequenced genomes reveals that all the Gram-positive rods and cocci as well as the mycoplasmas belong to a single clade that has been named the Firmicutes.

Gram-Positive Rods

Aerobic Gram-Positive Rods

- **Bacillus anthracis/cereus/thuringiensis**. These organisms differ mainly in the plasmids they contain.
- **B. anthracis** causes **anthrax**. Currently the biological agent favored by terrorists. Its 2 plasmids contain the genes needed to synthesize
  - a capsule which (like those of pneumococci) makes it resistant to phagocytosis
  - the three components of the **toxin** that causes the disease symptoms
- ***B. thuringiensis*** — the organism, its toxin, and even the gene (also plasmid-encoded) for the toxin are used as biocontrol agents against a variety of insect pests.
- ***Bacillus subtilis***. A common soil bacterium. Its chromosome contains 4,214,814 bp of DNA encoding 4,100 genes.
- **Lactobacillus**. Several species are used to convert milk into cheese, butter, and yogurt.

Anaerobic Gram-Positive Rods

- ***Clostridium tetani***. Clostridia are spore-forming obligate anaerobes. The spores of *C. tetani* are widespread in the soil and often get into the body through wounds. Puncture wounds (e.g., by splinters or nails) are particularly dangerous because they provide the anaerobic conditions needed for germination of the spores and growth of the bacteria.

  C. tetani liberates a **toxin** that blocks transmitter release (by destroying the SNAREs needed) at inhibitory synapses in the spinal cord and brain. This interferes with the reciprocal inhibition of antagonistic pairs of skeletal muscles so the victim suffers violent muscle spasms. Fortunately, the disease - called **tetanus -** is now rare in developed countries, thanks to almost universal immunization against the toxin. Chemical alteration of the toxin produces a **toxoid** that still retains the epitopes of the toxin. Incorporated in a vaccine, the toxoid provides a relatively long-lasting (~10 years) immunity against tetanus.

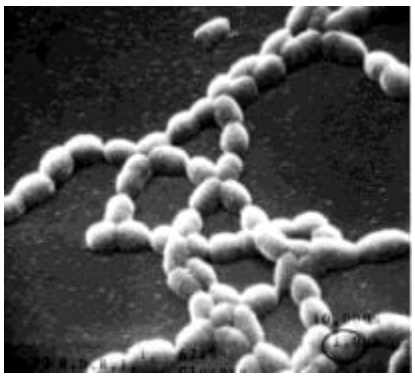


Figure 19.2.1.2 Strep

The bacteria in this group grow in characteristic colonies.

- Many cases of "food poisoning" are caused by staphylococci.
- Most **Streptococci** grow in chains. The electron micrograph (courtesy of the Naval Dental Research Institute, Great Lakes, IL) shows ***Streptococcus mutans***, a common inhabitant of the mouth.

  Streptococci cause

    - "strep throat"
    - impetigo
    - middle ear infections
    - scarlet fever (a result of a toxin produced by the organism)
    - rheumatic fever
    - a rare form of toxic shock syndrome
- **Pneumococci**. The cells of these streptococci grow in pairs. ***Streptococcus pneumoniae*** causes bacterial pneumonia. This was once a major killer — especially of the aged and infirm — but today there is an effective vaccine and any infections that do occur usually respond quickly to antibiotics.

Mycoplasmas

Mycoplasmas have the distinction of being the smallest living organisms. They are so small (0.1 µm) that they can be seen only under the electron microscope. Mycoplasmas are obligate parasites; that is, they can live only within the cells of other organisms. They are probably the descendants of Gram-positive bacteria who have lost their peptidoglycan wall as well as much of their genome — now depending on the gene products of their host.

The DNA sequences of the complete genomes of seven mycoplasmas have been determined, including

- Mycoplasma genitalium has 580,073 base pairs of DNA encoding 525 genes (485 for proteins; the rest for RNAs).
- ***Mycoplasma urealyticum*** has 751,719 base pairs of DNA encoding 651 genes (613 for proteins; 39 for RNAs).
- ***Mycoplasma pneumoniae*** has 816,394 base pairs of DNA encoding 679 genes.

How many genes does it take to make an organism?

The scientists at The Institute for Genomic Research (now known as the J. Craig Venter Institute - JCVI) who determined the **Mycoplasma genitalium** sequence followed this work by systematically destroying its genes (by mutating them with insertions) to see which ones are essential to life and which are dispensable. Of the 485 protein-encoding genes, they have concluded that only 381 of them are essential to life.

Workers at the JCVI have also succeeded in synthesizing the complete genome of one species of mycoplasma, inserted this into a second species, which converted the second species into the first.

Actinobacteria

Most of these **Gram-positive** organisms grow as thin filaments - like a mold - rather than as single cells. In fact, they were long thought to be fungi and were called actinomycetes. But fungi are eukaryotes and the actinobacteria are not.

Actinobacteria dominate the microbial life in soil where they play a major role in the decay of dead organic matter. Many of them have turned out to be the source of valuable antibiotics, including streptomycin, erythromycin, and the tetracyclines.

Mycobacteria and Corynebacteria

These **Gram-positive** organisms are closely related to the actinobacteria and often classified with them. They include three important human pathogens:

- Mycobacterium tuberculosis is the agent of tuberculosis (TB). TB is estimated to have killed 2 million people in 2007. Under ideal conditions, a single bacterium can cause infection. AIDS patients are especially at risk.

  Its genome contains 4,411,532 bp of DNA encoding some 3,959 genes.

- ***Mycobacterium leprae*** causes leprosy. Its genome contains 3,268,203 bp of DNA encoding only 1,604 genes.

  Although a close relative of *M. tuberculosis* (they share 1,439 genes), much of its DNA encodes pseudogenes, genes that no longer make a functional product. *M. leprae* is an obligate intracellular parasite; it has never been cultured in vitro. This is probably because it has abandoned many of the genes needed for an independent existence choosing instead to depend on the genes of its host cell.

- ***Corynebacterium diphtheriae*** causes diphtheria. As in tetanus, it isn't the growth of the organism (in the throat) that is dangerous but the toxin it liberates. The toxin is the product of a latent bacteriophage in the bacterium. It catalyzes the inactivation of a factor necessary for amino acids to be added to the polypeptide chain being synthesized on the ribosome. Sensibly enough, the toxin has no such effect on the translation machinery of bacteria (or of chloroplasts and mitochondria).

  Treatment of the toxin with formaldehyde converts it into a harmless **toxoid**. Immunization with this toxoid — usually incorporated along with tetanus toxoid and pertussis antigens in a "triple vaccine" (DTP) - protects against the disease.

Gram-negative Bacteria

The Proteobacteria

This large group of bacteria form a clade sharing related rRNA sequences. They are all **Gram-negative** but come in every shape (rods, cocci, spirilla). They are further subdivided into 5 clades: alpha-, beta-, gamma-, delta-, and epsilon proteobacteria.

Alpha (α) Proteobacteria.

Some examples:

- Rickettsias. These bacteria are too small to be clearly seen under the light microscope. Almost all are obligate intracellular parasites. This means that they can only grow and reproduce while within the living cells of their host - certain arthropods (ticks, mites, lice, fleas) and mammals.
  - **Rickettsia prowazekii** causes **typhus fever** when it is transmitted to humans by lice.
  - **Rocky Mountain spotted fever** is a rickettsial disease transmitted by ticks.

  The **mitochondria** of eukaryotes probably evolved from endosymbiotic bacteria. Because of the similarities of their genomes, rickettsias may be the closest relatives to the ancestors of mitochondria.
- **Rhizobia**. These bacteria live in a mutualistic relationship with the roots of legumes where they are able to "fix" nitrogen (N_2) in the air into compounds that can be used by living things.
- ***Magnetospirillum magnetotacticum***
- ***Agrobacterium tumefaciens***

Beta (β) Proteobacteria

- Sulfur bacteria.

  Certain colorless bacteria share the ability of chlorophyll-containing organisms to manufacture carbohydrates from inorganic raw materials, but they do not use light energy for this. These so-called **chemoautotrophic** bacteria secure the necessary energy by oxidizing some reduced substance present in their environment. The free energy released by the oxidation is harnessed to the manufacture of food.

  For example, some chemoautotrophic sulfur bacteria oxidize H_2S in their surroundings (e.g., the water of sulfur springs) to produce energy:

  $$2H_2S + O_2 \rightarrow 2S + 2H_2O;\ \Delta G = -100 \text{ kcal}$$

  They then use this energy to reduce carbon dioxide to carbohydrate (like the photosynthetic purple sulfur bacteria)

  $$2H_2S + CO_2 \rightarrow (CH_2O) + H_2O + 2S$$

- This **chemoautotroph** oxidizes NH_3 (produced from proteins by decay bacteria) to nitrites (NO_2^-). This provides the energy to drive their anabolic reactions. The nitrites are then converted (by other nitrifying bacteria) into nitrates (NO_3^-), which supply the nitrogen needs of plants.
- **Three important human pathogens among the β-proteobacteria.**
  - ***Neisseria meningitidis***.

Causes meningococcal meningitis, an extremely serious infection of the meninges that occasionally occurs in very young children and in military camps. There is a vaccine that is effective against several strains but unfortunately not the most dangerous one.

- ***Neisseria gonorrhoeae***. Causes **gonorrhea**, one of the most common sexually-transmitted diseases (**STDs**): over 300,000 cases were reported in the U.S. in 2009. In males, the bacterium invades the urethra causing a discharge of pus and often establishes itself in the prostate gland and epididymis. In females, it spreads from the vagina to the cervix and fallopian tubes. If the infection is untreated (penicillin is usually effective although strains resistant to it are now being encountered), the resulting damage to the fallopian tubes may obstruct the passage of eggs and thus cause sterility.
- ***Bordetella pertussis***; the cause of "whooping cough".

Gamma (γ) Proteobacteria

The largest and most diverse subgroup of the proteobacteria.

Some examples

- Escherichia coli. The most thoroughly-studied of all creatures (possibly excepting ourselves). Its entire genome has been determined down to the last nucleotide: 4,639,221 base pairs of DNA encoding 4,377 genes. Lives in the human colon, usually harmlessly. However, water or undercooked food contaminated with the **O157:H7** strain has caused severe - occasionally fatal - infections.
- ***Salmonella enterica***. Two major human pathogens:
  - ***Salmonella enterica* var Typhi**. Causes typhoid fever, a serious systemic infection occurring only in humans. This microbe is also known as ***Salmonella typhi***.
  - ***Salmonella enterica* var Typhimurium**. Confined to the intestine, it is a frequent cause of human gastrointestinal upsets but is also found in many other animals (that are often the source of the human infection). Also known as ***Salmonella typhimurium***.
- ***Vibrio cholerae***. Causes cholera, one of the most devastating of the intestinal diseases. The bacteria liberate a toxin that causes massive diarrhea (10–15 liters per day) and loss of salts. Unless the water and salts are replaced quickly, the victim may die (of shock) in a few hours. Like other intestinal diseases, cholera is contracted by ingestion of food or, more often, water that is contaminated with the bacteria.
- ***Pseudomonas aeruginosa***. A common inhabitant of soil and water, it can cause serious illness in humans with
  - defective immune systems
  - serious burns
  - cystic fibrosis

  Frequently encountered in hospitals and resistant to most antibiotics and disinfectants.
- ***Yersinia pestis***. This bacillus causes **bubonic plague**. It is usually transmitted to humans by the bite of an infected flea. As it spreads into the lymph nodes, it causes them to become greatly swollen, hence the name "bubonic" (bubo — swelling of a lymph node) plague. Once in the lungs, however, the bacteria can spread through the air causing the rapidly lethal (2–3 days) "pneumonic" plague. Untreated, ~30% of the cases of bubonic plague are fatal, and the figure for the pneumonic form reaches 100%. The recurrent epidemics of the "black death" in Europe from 1347–1351, which killed off at least 30% of the population, was caused by this organism. DNA sequencing of samples retrieved from the bodies of plague victims of that era confirm this diagnosis. Although no major epidemics have occurred in this century, the threat is not entirely over. ***Yersinia pestis*** still flourishes in some rodent populations in the western U. S. and causes a dozen or so cases of human plague - primarily among small game hunters -each year.
- ***Francisella tularensis*** causes **tularemia**. This is primarily a disease of small mammals, but about 100 people become infected each year in the United States. Most cases occur in south-central states (KS, MO, OK, AR). However, the import of infected rabbits by game clubs has introduced the disease to Cape Cod and Martha's Vineyard in Massachusetts. In the summer of 2000, 15 people became ill (one died) on the island. All seem to have acquired their infection as they used lawn mowers and brush cutters that presumably stirred up the organism from the carcasses of infected animals.
- ***Haemophilus influenzae*** was once thought to cause influenza. It does not, but it can cause bacterial meningitis and middle ear infections in children and pneumonia in adults — especially those whose resistance is lowered by other diseases (e.g., AIDS). There is now an effective vaccine against the most dangerous strains. The complete genome of Haemophilus influenzae is known: 1,830,138 bp of DNA encoding 1,743 genes.
- **Purple Sulfur Bacteria** Like green plants, these bacteria are photosynthetic, using the energy of sunlight to reduce carbon dioxide to carbohydrate. Unlike plants, however, they do not use water as a source of electrons.

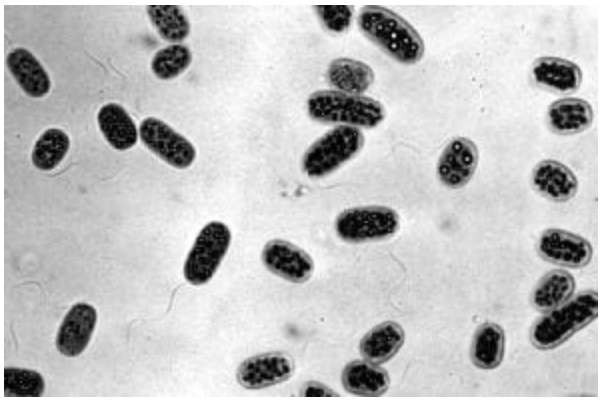


Figure 19.2.1.3 Chromatium

In the process, they produce elemental sulfur (often - as seen in this photomicrograph of **Chromatium -** stored as granules within the cell). [Image from H. G. Schlegel and N. Pfennig, *Arch. Microbiol.* **38**[1], 1961.]

Photosynthetic bacteria contain special types of chlorophylls (called bacteriochlorophylls) incorporated into membranes. With this machinery, they can run photosystem I but not photosystem II (which explains their inability to use water as a source of electrons). Most photosynthetic bacteria are obligate anaerobes; they cannot tolerate free oxygen. Thus they are restricted to such habitats as the surface of sediments at the bottom of shallow ponds and estuaries. Here they must make do with whatever radiant energy gets through the green algae and aquatic plants growing above them. However, the absorption spectrum of their bacteriochlorophylls lies mostly in the infrared region of the spectrum so they can trap energy missed by the green plants above them.

Delta (δ) Proteobacteria

This group contains the **myxobacteria**. They are found in vast numbers in soil and are major players in the decay of organic matter.

Epsilon (ε) Proteobacteria

Two members of this small group that are human pathogens:

- Helicobacter pylori, the main cause of stomach ulcers
- ***Campylobacter jejuni***; the bacterium most frequently implicated in gastrointestinal upsets.

Bacteroidetes



Figure 19.2.1.4 Spirochete

Two notorious examples:

- Treponema pallidum (right), the cause of **syphilis**, one of the most dangerous of the sexually transmitted diseases (STDs). (Image courtesy of Harry E. Morton.)
- ***Borrelia burgdorferi*** is transmitted to humans through the bite of a deer tick causing **Lyme disease** (over 30,000 cases — the largest number up to then — were reported in the U.S. in 2009).

Both these organisms have had their complete genomes sequenced.

Chlamydiae

Chlamydiae are also obligate intracellular parasites (they cannot make their own ATP).

- Its genome contains 1,042,519 bp of DNA encoding 894 genes. In 2008, over 1.2 million cases were reported in the U. S., and this is probably only half of the true total. The infection is usually spread by sexual intercourse making it the most common sexually-transmitted disease (STD). It is easily cured if diagnosed, but many infections remain untreated and, in females, are a major cause of **pelvic inflammatory disease**. This causes scarring of the uterus and fallopian tubes and often results in infertility.

  Mothers can pass the infection on to their newborn babies causing serious eye disease and pneumonia. To avoid this, pregnant women are usually tested for chlamydia and treated with antibiotics if they are infected.

- **Chlamydia psittaci** usually infects birds, but can infect their human contacts causing **psittacosis** (a.k.a. ornithosis).

Cyanobacteria (blue-green algae)

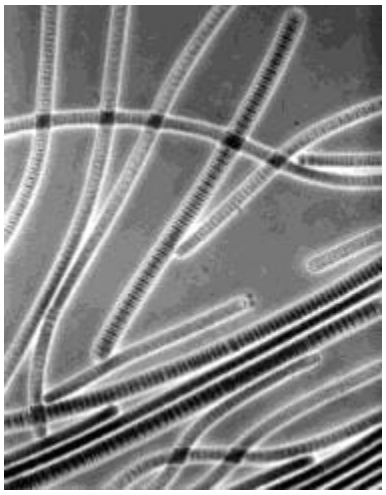


Figure 19.2.1.5 Oscillatoria

Unlike other photosynthetic bacteria, cyanobacteria

- use **chlorophyll a** (as do plants)
- use water as the source of electrons to reduce CO_2 to carbohydrate (because they have photosystem II as well as photosystem I).

$$CO_2 + 2H_2O \rightarrow (CH_2O) + H_2O + O_2$$

It is estimated that cyanobacteria are responsible for ~ 25% of the photosynthesis occurring on our planet.

The micrograph is of **Oscillatoria**, a filamentous cyanobacterium (magnified about 800 times). Each disk in the chains is one cell.

Cyanobacteria also contain two antenna pigments:

- blue **phycocyanin** (making them "blue-green")
- red **phycoerythrin** (The Red Sea gets its name from the periodic blooms of red-colored cyanobacteria.)

These two pigments also occur in red algae. Their chloroplasts (in fact probably all chloroplasts) evolved from an endosymbiotic cyanobacterium.

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19.2B: Archaea

When these microscopic organisms were first discovered (in 1977), they were considered bacteria. However, when their ribosomal RNA was sequenced, it became obvious that they bore no close relationship to the bacteria and were, in fact, more closely related to the eukaryotes (including ourselves!) For a time they were referred to as archaebacteria, but now to emphasize their distinctness, we call them Archaea. They have also been called **Extremophiles** in recognition of the extreme environments in which they have been found:

- **thermophiles** that live at high temperatures
- **hyperthermophiles** that live at really high temperatures (present record is 121°C!)
- **psychrophiles** that like it cold (one in the Antarctic grows best at 4°C)
- **halophiles** that live in very saline environments (like the Dead Sea)
- **acidophiles** that live at low pH (as low as pH 1 and who die at pH 7!)
- **alkaliphiles** that thrive at a high pH.

Most of the >250 named species that have been discovered so far have been placed in two groups: Euryarchaeota and Crenarchaeota

Euryarchaeota

There are three main groups: Methanogens, Halophiles. and Thermoacidophiles.

Methanogens

These are found living in such anaerobic environments as

- the muck of swamps and marshes
- the rumen of cattle (where they live on the hydrogen and CO_2 produced by other microbes living along with them)
- our colon (large intestine)
- sewage sludge
- the gut of termites

They are chemoautotrophs; using hydrogen as a source of electrons for reducing carbon dioxide to food and giving off methane ("marsh gas", CH_4) as a byproduct.

$$4\,H_2 + CO_2 \longrightarrow CH_4 + 2\,H_2O$$

Two methanogens that have had their complete genomes sequenced:

- ***Methanocaldococcus jannaschii***
- ***Methanothermobacter thermoautotrophicus***

Halophiles

These are found in extremely saline environments such as the Great Salt Lake in the U.S. and the Dead Sea. They maintain osmotic balance with their surroundings by building up the solute concentration within their cells.

Thermoacidophiles

As their name suggests, these like it hot and acid (but not as hot as some of the Crenarchaeota!). They are found in such places as acidic sulfur springs (e.g., in Yellowstone National Park) and undersea vents ("black smokers").

Crenarchaeota

The first members of this group to be discovered like it really hot and so are called **hyperthermophiles**. One can grow at 121°C (the same temperature in the autoclaves used to sterilize culture media, surgical instruments, etc.). Many like it acid as well as hot and live in acidic sulfur springs at a pH as low as 1 (the equivalent of dilute sulfuric acid). These use hydrogen as a source of electrons to reduce sulfur in order to get the energy they need to synthesize their food (from CO_2).

Aeropyrum pernix is one member of the group that has had its genome completely sequenced. Other members of this group seem to make up a large fraction of the plankton in cool, marine waters and the microbes in both soil and the ocean that convert ammonia into nitrites (nitrification).

Evolutionary Position of the Archaea

The archaea have a curious mix of traits characteristic of bacteria as well as traits found in eukaryotes. The table summarizes some of them.

Eukaryotic Traits	Bacterial Traits
• DNA replication machinery • histones • nucleosome-like structures • Transcription machinery ◦ RNA polymerase ◦ TFIIB ◦ TATA-binding protein (TBP) • Translation machinery ◦ initiation factors ◦ ribosomal proteins ◦ elongation factors ◦ poisoned by diphtheria toxin	• single, circular chromosome • operons • no introns • bacterial-type membrane transport channels • Many metabolic processes ◦ energy production ◦ nitrogen-fixation ◦ polysaccharide synthesis

What can we conclude from this collection of traits?

Many traits found in the bacteria first appeared in the ancestors of all the present-day groups. The split leading to the archaea and the eukaryotes occurred after the bacteria had gone their own way. However, the acquisition by eukaryotes of mitochondria (probably from an ancestor of today's rickettsias) and chloroplasts (from cyanobacteria) occurred after their line had diverged from the archaea (i.e., the endosymbiosis hypothesis).

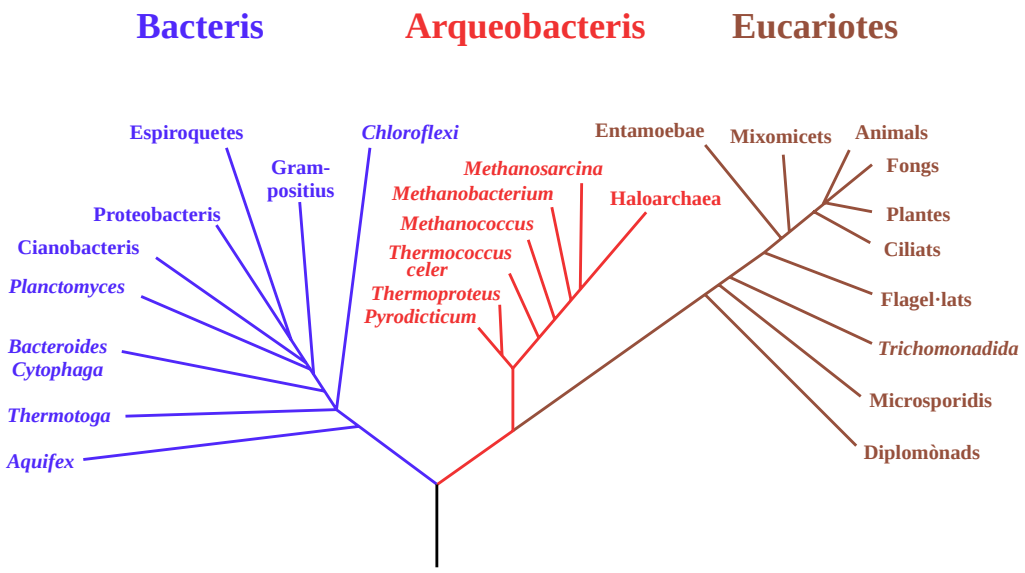


Figure 19.2.2.1 Tree of life A speculatively rooted tree for rRNA genes, showing the three life domains Bacteria, Archaea, and Eukaryota, and linking the three branches of living organisms to the LUCA (the black trunk at the bottom of the tree), 2009. (Public Domain; NASA Astrobiology Institute via Wikipedia)

As more and more genes are sequenced, it appears that the line that eventually produced eukaryotes split off **after** the line leading to the euryarchaeota. If that is the case, Archaea is a paraphyletic group, and we shared a common ancestor with the other archaea more recently than they (and we) did with the euryarchaeota.

Economic Importance of the Archaea

Because they have enzymes that can function at high temperatures, considerable effort is being made to exploit the archaea for commercial processes such as providing enzymes to be added to detergents (maintain their activity at high temperatures and pH) and an enzyme to covert corn starch into dextrins. Archaea may also be enlisted to aid in cleaning up contaminated sites, e.g., petroleum spills.

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19.2C: Antibiotics

Humans, and our domestic animals, can serve as hosts to a wide variety of disease-causing organisms (**pathogens**) including bacteria, viruses, fungi, protozoans, helminths (worms). This page will examine only those chemical agents that are used to combat bacterial pathogens.

The Problem

There are many chemicals that are lethal to bacteria - cyanide does a good job — but they cannot be used to cure infections because they are lethal to the host as well. The problem, then, is to find substances that attack a metabolic pathway found in the bacterium but not in the host. This is not an insurmountable problem for bacterial pathogens because they differ in many respects from eukaryotes.

The Solution

Natural products. A number of natural products, penicillin for example, have been discovered that are antibiotics suitable for therapy. They were originally discovered as secretions of fungi or soil bacteria. Soils are complex ecosystems, and it is not surprising that its inhabitants have evolved chemical defenses against each other.

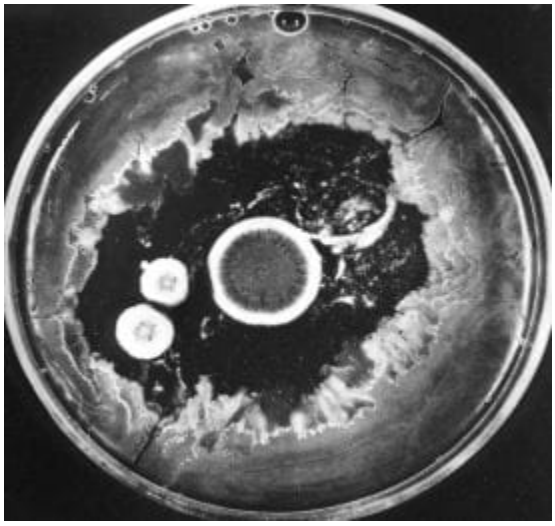


Figure 19.2.3.1 Penicillium

The photo (courtesy of Merck & Co., Inc.) shows how the growth of bacteria on the agar in a culture dish has been inhibited by the three circular colonies of the fungus ***Penicillium notatum***. The antibiotic penicillin, diffusing outward from the colonies, is responsible for this effect. Today, penicillin is made from cultures of ***Penicillium chrysogenum*** that has been specially adapted for high yields.

- **Semi-synthetic products.** These are natural products that have been chemically modified in the laboratory (and pharmaceutical facility) to
  - improve the efficacy of the natural product
  - reduce its side effects
  - circumvent developing resistance by the targeted bacteria
  - expand the range of bacteria that can be treated with it
- **Completely synthetic products.** The sulfa drugs are examples.

Sulfa Drugs and Folic Acid Analogs

Sulfa Drugs

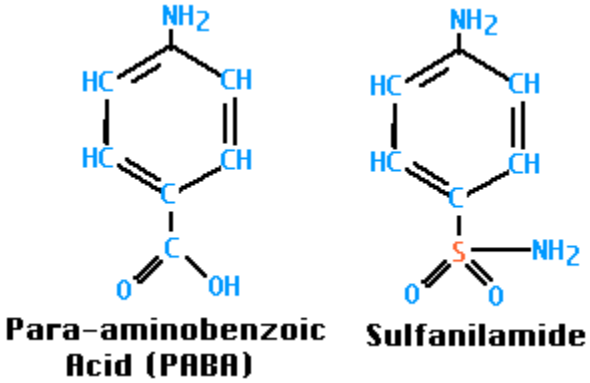


Figure 19.2.3.2 PABA

Sulfanilamide was the first antibacterial agent. Many other sulfa drugs (such as sulfamethoxazole) have since come into use.

> Both bacteria and their human hosts require folic acid for nucleic acid synthesis (it is converted into purines and thymidine) as well as protein synthesis (precursor of the amino acids methionine and glycine). However, bacteria **synthesize** their folic acid starting with **para-aminobenzoic acid** (**PABA**), while we must ingest our folic acid already formed; that is, for us it is a **vitamin**.
>
> Sulfanilamide, and the other sulfa drugs, are analogs of PABA; they compete with PABA and, when chosen, block the synthesis of folic acid. Mammals ignore PABA and its analogs and thus can tolerate sulfa drugs.

Folic Acid Analogs

These synthetic molecules block the final step in the conversion of PABA to folic acid so they, too, block nucleotide and protein synthesis in bacteria but not in mammals. **Trimethoprim** is one of several in current use. These folic acid analogs are often used in combination with a sulfa drug.

The Beta-Lactams

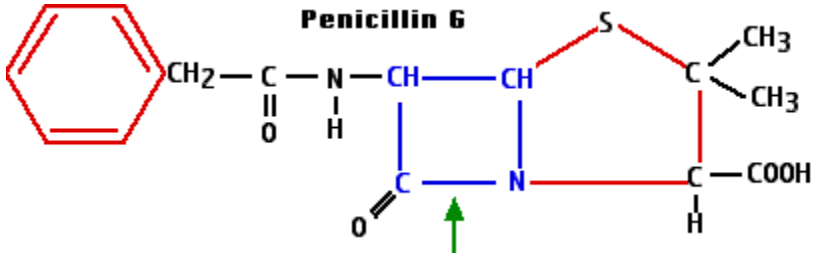


Figure 19.2.3.3 Penicillin G. The beta-lactams get their name from the characteristic ring structure - shown here in blue - that they all share. (The green arrow shows the bond that is broken by the beta-lactamases that are synthesized by many penicillin-resistant bacteria.)

The beta-lactams include the

- **penicillins** such as
  - penicillin G (a natural product) produced by the fungus ***Penicillium chrysogenum***
  - ampicillin (a semi-synthetic)
  - amoxicillin (semi-synthetic)
- **cephalosporins** There are over two dozen of them in current use. Most are semi-synthetics derived from the secretion of the mold Cephalosporium. Some examples:
  - cephalexin (e.g., Keflex®)

- cefaclor (e.g., Ceclor®)
- cefixime (e.g., Suprax®)

- **carbapenems** such as
  - meropenem (Merrem®)
  - ertapenem (Invanz®)

> The beta-lactams all work by interfering with the synthesis of the bacterial cell wall — a structure that is not found in eukaryotes. The walls of bacteria are made of a complex polymeric material called **peptidoglycan**.

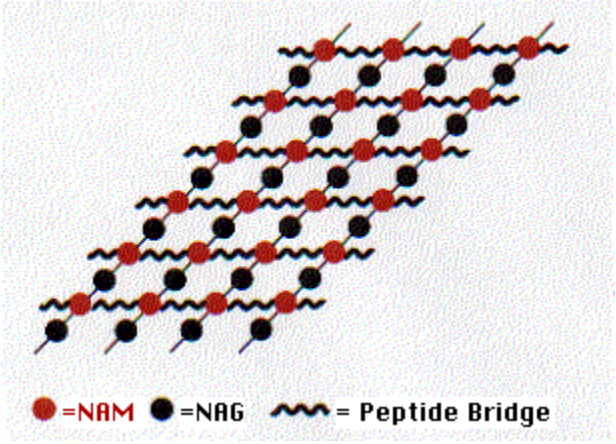


Figure 19.2.3.4 NAG - NAM

Peptidoglycan contains both amino acids and amino sugars. The amino sugars are of two kinds

- **N-acetylglucosamine** (**NAG**) and its close relative
- **N-acetylmuramic acid** (**NAM**).

These two form a linear polymer of NAG alternating with NAM. They are linked by a **glycosidic bond** between the #1 and #4 carbons (this is the linkage attacked by lysozyme) and are oriented in the same way they are in cellulose. Side chains containing 4 or 5 amino acids are attached to each NAM. These form covalent bonds with amino acids in adjacent chains. The bonds may be direct to the next chain or include additional **peptide cross bridges** (e.g., 5 glycine residues) which extend to chains in the same plane (shown here) as well as to chains above and below.

This elaborate, covalently cross-linked structure provides the great strength of the cell wall. It also leads to the remarkable conclusion that the bacterial cell wall meets the definition of a single molecule!

The beta-lactam antibiotics bind to and inhibit enzymes needed for the synthesis of the peptidoglycan wall. While they have little effect on resting bacteria, they are lethal to dividing bacteria as defective walls cannot protect the organism form bursting in hypotonic surroundings.

Aminoglycosides

These are products of actinomycetes (soil bacteria) or semi-synthetic derivatives of the natural products.

Examples are:

- streptomycin
- kanamycin
- neomycin
- gentamycin

The 70S bacterial ribosome differs in several ways from the 80S eukaryotic ribosome. The aminoglycosides bind to the 30S subunit of the bacterial ribosome and interfere with the formation of the initiation complex. They also cause misreading of the mRNA. Although the eukaryotic ribosome in the cytosol is relatively unaffected by these drugs, ribosomes in the mitochondria are 70S and sensitive to their effects.

Tetracyclines

These are natural products derived from soil actinomycetes or their semi-synthetic derivatives. Examples:

- chlortetracycline (aureomycin®)
- oxytetracycline (terramycin®)
- doxycycline
- tigecycline (Tygacil®)

Tetracyclines bind to the **30S** subunit of the bacterial ribosome. They prevent the transfer of activated amino acids to the ribosome so protein synthesis is halted.

Macrolides, Lincosamides, Streptogramins, and Ketolides

The Chink in the Armor = the bacterial ribosome

These antibiotics bind to the large (**50S**) subunit of the bacterial ribosome where they block the growing peptide chain from exiting the ribosome thus severely hindering protein synthesis. Because of their similar action, the development of antibiotic resistance to one usually extends to all the others.

Macrolides

Macrolides are also products of actinomycetes (soil bacteria) or semi-synthetic derivatives of them. **Erythromycin**, azithromycin (Zithromax®), and **clarithromycin** (Biaxin®) are a commonly-prescribed macrolides.

Lincosamides

The first member of this group was also isolated from a soil actinomycete (found near Lincoln, Nebraska). A semi-synthetic derivative, called **clindamycin** (Cleocin®), is now widely used against Gram-positive bacteria.

Streptogramins

Quinupristin and **dalfopristin** are examples. As of 1 October 1999, they will be sold as a mixture under the trade name **Synercid**. Combined, they show great promise in treating certain infections resistant to vancomycin- currently the antibiotic of last resort for some hospital-acquired infections.

Ketolides

Ketolides are derivatives of macrolides. Telithromycin (Ketek®) is an example. Ketolides also bind to the 50S subunit of the bacterial ribosome. In so doing they cause it to induce frameshifts during translation. Bacteria that have become resistant to macrolides, lincosamides, and streptogramins are still susceptible to ketolides.

Fluoroquinolones

Ciprofloxacin (Cipro®), levofloxacin and norfloxacin are examples. Cipro is the preferred antibiotic for people who have been intentionally exposed to anthrax, although some other antibiotics appear to be equally effective.

The Chink in the Armor = DNA topoisomerases

The fluoroquinolones block the action of two bacterial topoisomerases - enzymes that relieve the coils that form in DNA when the helix is being opened in preparation for replication or transcription or repair. The topoisomerases in eukaryotes are not affected.

Polypeptides

The most common of these is **polymixin E** (also known as colistin). It behaves as a detergent, increasing the permeability of the membranes that encase bacteria and causing the contents of the bacterial cell to leak out.

Rifampin

This semi-synthetic antibiotic binds to the bacterial **RNA polymerase** and prevents it from carrying out its role in transcription. Its affinity for the equivalent eukaryotic enzyme is much lower. Rifampin is also known as rifampicin.

Mupirocin

This antibiotic blocks the action of the bacterial **isoleucine tRNA synthetase**, the enzyme responsible for attaching the amino acid isoleucine (Ile) to its tRNA in preparation for protein synthesis, so protein synthesis is inhibited. It spares the equivalent eukaryotic enzyme.

Cycloserine

Cycloserine inhibits synthesis of the bacterial cell wall but by a different mechanism than the beta-lactam antibiotics discussed above. Cycloserine is an analog of D-alanine and blocks the incorporation of D-alanine into the peptide bridges in the bacterial cell wall. It is derived from an actinomycete.

Aminocyclitols

These products of another actinomycete achieve their effect by interfering with the **30S** subunit of the bacterial ribosome. **Spectinomycin** (trade name = Trobicin®) is an example. It is particularly effective against the gonococcus, the bacterium that causes the sexually-transmitted disease (STD) gonorrhea.

Glycopeptides

Glycopeptides also interfere with the synthesis of the bacterial cell wall but by a different mechanism than the beta-lactams.

Vancomycin is a widely-used glycopeptide in the U.S. It binds to the **D-alanines** on the precursors of the peptidoglycan cross bridges preventing their cross-linking. It has become the antibiotic of last resort as resistance to the other antibiotics has become more and more common.

Oxazolidinones

The first of these new antibiotics, **linezolid** (**Zyvox®**), was approved by the U.S. Food and Drug Administration on 19 April 2000. It is effective against many Gram-positive bacteria that have developed resistance to the older antibiotics.

Linezolid attacks a previously-unexploited chink in the bacterium's armor: the proper assembly of the two ribosomal subunits (30S and 50S). It does not affect eukaryotic ribosomes — and thus translation of mRNAs in the cytosol. However, it does affect the bacterial-like mitochondrial ribosomes and can interfere with the synthesis of those mitochondrial proteins synthesized by them.

Lipopeptides

These are natural compounds derived from a species of Streptomyces. The one now in clinical use is daptomycin (Cubicin®). It

is effective against Gram-positive bacteria. It attacks another previously-unexploited chink in the bacterial armor — the integrity of its cell membranes.

So far there is no evidence of bacteria developing resistance against it.

Resistance to Antibiotics

None of the antibiotics discussed above is effective against all bacterial pathogens.

Intrinsic resistance

Some bacteria are intrinsically resistant to certain of the antibiotics. Example: Gram-positive bacteria are much less susceptible to polymixins than Gram-negative bacteria. [The "Gram" designations refer to the behavior of the bacteria when stained with the Gram stain; this behavior is a reflection of the very different organization of their cell walls.]

Acquired resistance

Many bacteria acquire resistance to one or more of the antibiotics to which they were formerly susceptible.

Example: In the U.S. in the decade from 1985–1995, resistance of Shigella (which causes gastrointestinal illness) to ampicillin grew from 32% to 67%. And, while only 7% of these isolates were resistant to the combination of sulfamethoxazole and trimethoprim at the start of the decade, that figure had grown to 35% by the end of the decade.

Bacteria develop resistance by acquiring genes encoding proteins that protect them from the effects of the antibiotic. In some cases the genes arise by mutation; in others, they are acquired from other bacteria that are already resistant to the antibiotic. The genes are often found on plasmids which spread easily from one bacterium to another - even from one species of bacterium to another.

Examples:

- Synthesis of the enzyme penicillinase - or other **beta-lactamases -** provides protection from the beta-lactam antibiotics. These enzymes break the beta-lactam ring at the position shown with the green arrow in the diagram of penicillin G.
- Likewise synthesis of **cephalosporinases** defeats the cephalosporins.
- Defeating quinolones:
  - Some bacteria do this by modifying their **DNA gyrase**.
  - Others, e.g., *Mycobacterium tuberculosis*, develop quinolone resistance by synthesizing a protein that resembles a short length of DNA. This protein binds the gyrase so it cannot form the DNA/gyrase complex that is the target of quinolone action.
- Some bacteria synthesize "pumps" in their plasma membrane through which they remove antibiotics like tetracyclines from the interior of the cell.
- Bacteria may methylate their ribosomes obscuring the target of antibiotics (e.g., erythromycin) that ordinarily bind to and inactivate the ribosome — or conversely
- they may enzymatically modify the antibiotic (e.g., kanamycin) so it can no longer "see" its ribosomal target.
- Bacteria may modify the structure of their **peptidoglycan wall** and thus avoid the inhibitory effects of antibiotics like cycloserine.

An alarming number of human pathogens have acquired genes to combat **all** the presently-used antibiotics except vancomycin and recently vancomycin-resistant bacteria have appeared. These multidrug-resistant strains are particularly common in hospitals where antibiotic use is heavy, and the patients often have weakened immune systems.

Measuring Antibiotic Resistance

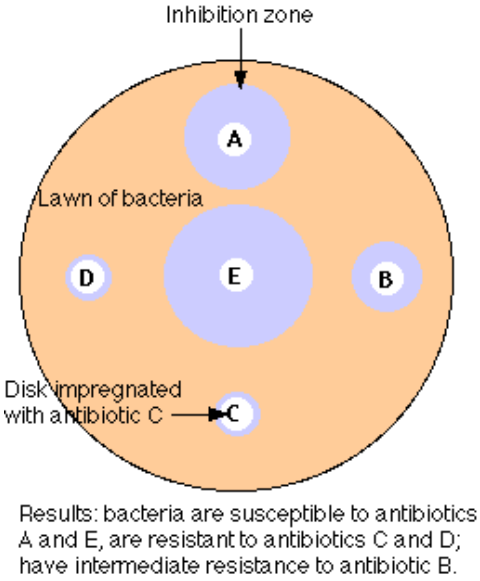


Figure 19.2.3.5 Antibiotic disks

The figure illustrates the simplest method of the several available for measuring antibiotic resistance.

- A suspension of the bacteria to be tested (e.g. cultured from the infected patient) is spread over the surface of a petri dish containing a solid culture medium.
- Disks of several different antibiotics are pressed on the surface of the agar. The concentration of antibiotic in each type of disk is standardized.
- Incubate overnight.
- The bacteria will grown into a "lawn" except where an antibiotic to which they are sensitive has diffused out from its disk.
- Measure the diameter of any zones of inhibition that are formed.

What can you do to delay the spread of antibiotic resistance?

- Don't ask your doctor for an antibiotic to treat a viral disease (e.g., a cold) for which antibiotics are useless. (However, your doctor may prescribe an antibiotic if you are infected by an influenza virus - not to fight the virus but to protect you against a secondary bacterial infection of your damaged lungs.)
- Stay the course. Use all doses prescribed even though you are feeling better. This will minimize the opportunity to select for resistance among the bacteria that remain late in the infection.
- Don't save unused antibiotics for later self-medication.

Farmers can help as well by avoiding the use of antibiotics in their livestock that are similar to those used in humans. Antibiotics are widely used in healthy livestock to improve their growth rate (by an unknown mechanism).

An article in the 20 May 1999 issue of **The New England Journal of Medicine** documents the recent development of **quinolone** resistance in ***Campylobacter jejuni***, the most frequent bacterial cause of gastroenteritis in humans. The rise coincides with the approval in 1995 of the use of quinolones by U. S. poultry farmers (chickens also become infected by *C. jejuni*). Similar recent increases in fluoroquinolone-resistant *C. jejuni* have been reported in the Netherlands and also in Spain (where as many as 50% of human infections are now caused by bacteria resistant to the antibiotic). In each country, the appearance of resistant strains followed the widespread introduction of quinolone treatment for animals.

Future Prospects

Drug companies - after many years of complacency - are now responding to the threat of antibiotic-resistant bacteria. Over a dozen new antibiotics are being developed and some have already reached clinical trials. Many of these are semi-synthetic modifications of already-existing antibiotics, including new

- beta-lactams
- macrolides
- glycopeptides
- quinolones
- modifications of vancomycin

Others are entirely new, attacking previously-unexploited chinks in the bacterial armor.

- Urea hydroxamates, that block the enzyme (peptide deformylase) that removes fMet from the finished protein so it can begin its work. (Eukaryotes do not begin translation with fMET.)
- Heteroaromatic polycycles (HARP) that bind to bacterial promoters preventing gene transcription.

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19.2D: E. coli

Escherichia coli is a bacterium that is a common - but certainly not the most abundant - inhabitant of the human colon. It also lives in the intestine of many other animals, wild as well as domestic.

Normally, *E. coli* does not cause disease although some strains frequently cause diarrhea in travelers, and it is the most common cause of urinary tract infections. One strain, designated **O157:H7**, is particularly virulent and has been responsible for several dangerous outbreaks in people eating contaminated food (usually undercooked hamburger).

Drinking water is tested for the presence of *E. coli* and related bacteria not because these bacteria are particularly dangerous but because they are an indication of contamination by sewage, and sewage may contain organisms (e.g., Salmonella, hepatitis A virus) that are dangerous.

E. coli is one of the most thoroughly studied of all living things. It is a favorite organism for genetic engineering as cultures of it can be made to produce unlimited quantities of the product of an introduced gene. Several important drugs (insulin, for example) are now manufactured in *E. coli*. However, *E. coli* cannot attach sugars to proteins so proteins requiring such sugars (e.g., glycoprotein hormones and clotting factors) have to be made in the cells of eukaryotes such as yeast cells and mammalian cells grown in cell culture.

Because *E. coli* lives in the human intestine, this has raised fears that genetically-engineered versions might escape from the laboratory (or factory) and take up residence in humans, producing a product that might be harmful. For this reason, genetic engineering is done only on strains of *E. coli* that have been deliberately weakened so that they cannot survive for long in humans.

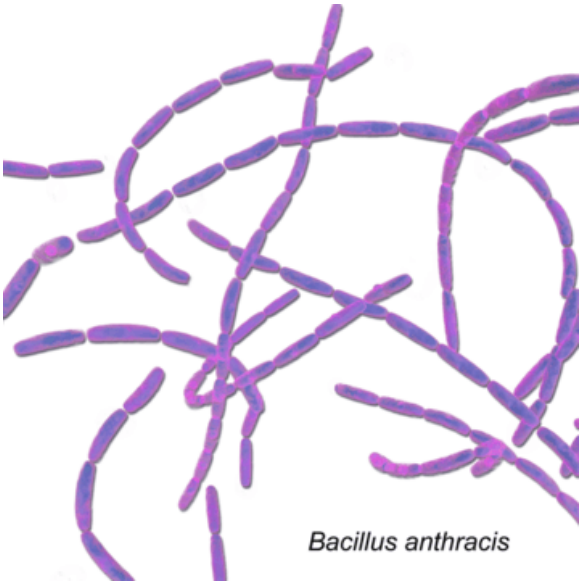
The complete sequence of the genome of a harmless laboratory strain of *E. coli* (K-12) was reported in the 5 September 1997 issue of **Science**. The genome consists of a single molecule of DNA containing 4,639,221 base pairs. These encode 4288 proteins and 89 RNAs. Many of the genes were already known and the function of many others can be deduced from the similarity to known genes.

The complete sequence of the pathogenic strain **O157:H7** was reported in the 25 January 2001 issue of **Nature**. It contains 5416 genes in 5.44×10^6 base pairs of DNA. Remarkably, these include 1,387 genes that are not present in its harmless laboratory relative *E. coli* K-12 (and K-12 has 528 genes that are not found in O157:H7). So here are two strains of the same species that differ in some 25% of their genes. Compare this with the difference between the genomes of humans and chimpanzees which probably is no more than 1%!)

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19.2E: Anthrax

Anthrax is a disease caused by the bacterium ***Bacillus anthracis***. It normally affects cattle, sheep, goats, etc. It is acquired from spores that remain viable in the environment (e.g. soil) for decades always able to germinate into active bacteria if they get into the body of a susceptible host. Humans can be infected by *Bacillus anthracis*, and if the spores enter by way of the lungs, the disease can be quickly fatal. These properties have made it one of the agents favored by bioterrorists.



Bacillus Anthracis. (CC BY-SA 4.0; BruceBlaus).

It is not the tissue-destructiveness of the active bacteria that is the problem but rather the **toxin** that they secrete. (This is like the illnesses caused by the bacilli that cause diphtheria, tetanus, and botulism.) The bacteria are susceptible to antibiotics; for example, Ciprofloxacin (Cipro®) is effective as are others such as doxycycline, but they must be given early before the toxin can produce symptoms. Once the toxin is in the system, it can be neutralized by giving **antitoxin** antibodies (conferring passive immunity). Presently these are harvested from donors who had received anthrax vaccine in the armed forces. But there is hope that monoclonal antibodies can be manufactured that will be able to provide protection.

The **anthrax toxin** is composed of three different proteins (encoded by genes on one of the two plasmids in the organism):

- **PA** ("protective antigen") It gets this name because it provides the epitopes that elicit protective antibodies in the anthrax vaccine.
- **LF** ("lethal factor")
- **EF** ("edema factor")

Infection

- PA molecules bind to receptors at the cell surface assembling in clusters of 7.
- LF and/or EF molecules then bind to these clusters.
- The complex is engulfed by receptor mediated endocytosis.
- The drop in pH in the endosome (endocytic vesicle) produces a change in the structure of the PA cluster enabling it to release its LF and EF into the cytosol.
- EF is an adenylyl cyclase which raises the intracellular concentration of cAMP inhibiting phagocytosis by neutrophils.
- As it name implies, LF in the cytosol so disturbs the machinery of the cell that it dies.

Future Prospects

In April of 2001, John Collier and his colleagues reported that several mutant versions of PA protected rats from death by the active anthrax toxin. The mutant molecules coassembled with normal PA molecules, and the complex was still able to bind LF and EF. However, the complex could not release LF or EF from the endosome, and the rats remained healthy. Perhaps one of these altered PA molecules can be enlisted in the fight against bioterrorism as both a vaccine to protect people **before** exposure and a treatment to block the lethal effects of the natural toxin **after** exposure.

Today's Anthrax Vaccine

The vaccine is made from an extract of a weakened strain of *B. anthracis* (it makes no surface capsule). The extract contains large amounts of PA, as well as some LF and EF.

Anthrax Strains

Scores of different strains of anthrax have been isolated from many parts of the world. While they share most of their harmful traits, their genomes differ in the number of repeated sequences of noncoding DNA. These are called **VNTR**s (for variable number of tandem repeats) and are simply longer versions of the short tandem repeats (STRs) now being used by law enforcement agencies for DNA fingerprinting of humans. DNA fingerprinting of any strain of anthrax used by bioterrorists may help track down its source.

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19.2F: Bacillus Thuringiensis

Bacillus thuringiensis is a bacterium that parasitizes the caterpillars of some harmful moths and butterflies. Spraying or dusting plants with spores of this bacterium appear to be environmentally safe ways to attack such pests as the gypsy moth, the tent caterpillar, and the tobacco hornworm (which also attacks tomatoes). The bacteria kill by a toxin which they secrete. The gene for this toxin has been introduced into some crop plants in an effort to protect them from insect attack without the need for spraying.



Figure 19.2.6.1 Bollworm. Image courtesy of the Monsanto Corp.

On the left is a cotton boll being attacked by a cotton bollworm. The cotton plant that produced the boll on the right contains and expresses the gene for the Bt toxin. The gene was introduced into the plant by genetic engineering. Transgenic cotton and corn (maize) containing the gene for Bt toxin were widely planted for the first time in 1996. By 2009, these crops had been planted on over 334 million acres (135×10^6 hectares) worldwide. In the United States over 70% of the cotton planted and 63% of the corn planted is now with transgenic varieties. Crops transgenic for Bt toxin have shown improved yields with greatly-reduced need for chemical insecticides. Not only does this reduce costs and possible health effects, but spares the natural enemies of other insect pests.

B. thuringiensis appears to be the same organism as ***Bacillus cereus***, a usually harmless soil organism, and ***Bacillus anthracis***, the cause of anthrax. What gives them their distinctive properties are the genes encoded by the plasmids they contain.

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19.2G: The Rapid Identification of Microorganisms

This page highlights the importance of diagnosing infections and food safety testing to identify harmful organisms. It reviews detection methods including traditional culturing, PCR for rapid but contamination-sensitive results, and longer immunoassays. A new rapid biosensor, CANARY, is introduced, which uses genetically engineered B lymphocytes to detect antigens, emitting light when specific antigens are present. This method is noted for its high sensitivity, specificity, and speed.

The Need

- diagnosis of infection so that the appropriate treatment (e.g., an antibiotic) can be started.
- testing of food to ensure that it is not contaminated with infectious organisms like *E. coli* O157:H7 and *Salmonella enterica*
- to identify the biological agent such as anthrax and smallpox in a possible terrorist attack so that appropriate measures can be taken quickly

Methods

Culturing

- The oldest and still most common.
- For bacteria, spread samples on culture media and examine the resulting colonies for morphology and metabolic traits. For viruses, inoculate cultures of living cells.
- Disadvantage: it make take several days to learn the results.

Polymerase Chain Reaction (PCR)

- Extract DNA from the sample and perform PCR.
- Advantage: rapid (often less than an hour)
- Disadvantage: overly sensitive to presence of contaminants

Immunoassays

Use a method that exploits the specificity and sensitivity of the reaction between antigen and antibodies. Takes 15 minutes or longer.

Biosensors (CANARY)

In the 11 July 2003 issue of **Science**, a team of scientists at the Lincoln Laboratory in the U. S. reported a new method of rapid identification that exploits living cells. They call their method CANARY (for **C**ellular **A**nalysis and **N**otification of **A**ntigen **R**isks and **Y**ields)

Their "biosensor" is a clone of B lymphocytes (B cells) that have been genetically engineered to express

- a B cell receptor for antigen (BCR) selected to interact with an epitope on the suspected agent. The BCR on their clones is surface IgM.
- aequorin, a protein extracted from the same jellyfish that produces green fluorescent protein.
    - Aequorin emits light when it is exposed to calcium ions (Ca^{2+}).
    - One of the first events (within seconds) when BCRs bind to antigen is a rise in the level of calcium ions in the cytosol.

Procedure:

- Prepare the sample.
- Mix - in separate wells - with B-cell clones each specific for a different suspected agent.

- Place in a sensitive light detector.
- If a clone has a BCR for an epitope present in the sample, that clone will emit light within a few seconds.

Results:

- highly sensitive: can detect as few as 50 bacteria or 500 virions
- highly specific: can detect the agent even in the presence of related contaminating agents.
- fast: time elapsed from sample preparation to signal from the light detector is often less than 5 minutes.

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19.3: VIRUSES

19.3A: Viruses
19.3B: Influenza
19.3C: φX174
19.3D: Smallpox
19.3E: Retroviruses

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19.3A: Viruses

Viruses are obligate intracellular parasites. Probably there are no cells in nature that escape infection by one or more kinds of viruses. Viruses that infect bacteria are called **bacteriophages**. Outside the cell, they consist of particles called **virions**.

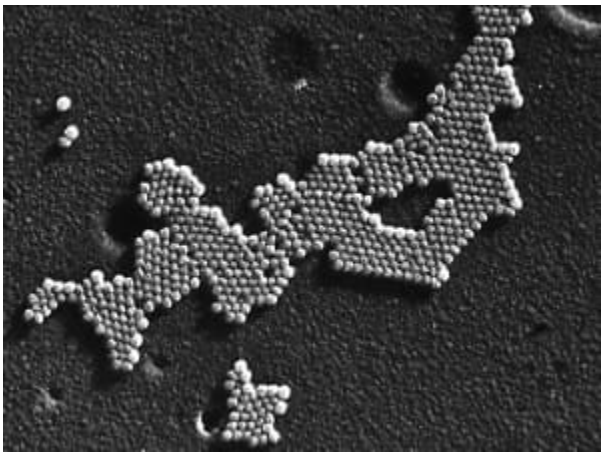


Figure 19.3.1.1 Poliovirus

Virions range in size from as small as the **poliovirus** shown above magnified some 450,000 times (courtesy of A. R. Taylor), which is 30 nm in diameter (about the size of a ribosome) to as large as *Pithovirus sibericum* an amoeba-infecting virus which, at 1,500 nm, is larger than many bacteria.

- The virion consists of
  - An outer shell, the **capsid**, made of protein. The capsid is responsible for
    - protecting the contents of the core
    - establishing what kind of cell the virion can attach to infecting that cell

    Some capsids contain other ingredients (e.g., lipids, carbohydrates), but these are derived from their host cells.
  - an interior core containing
    - the **genome**; either **DNA** or **RNA** The genes are few in number (3–100 depending on the species). They encode those proteins needed for viral reproduction that the **host cell will not supply**.
    - Often, one or more **proteins** (enzymes) needed to start the process of reproduction within the host cell.

Life Cycle

- The virion attaches to the surface of the host cell - usually binding to a specific cell surface molecule that accounts for the specificity of the infection. Example: **HIV-1**, the cause of **AIDS**, binds to the chemokine receptor **CCR5** found on human lymphocytes and macrophages.
- Once inside the cell, the virions are uncoated.
- Viral genes begin to be expressed leading to the synthesis of proteins needed for
  - replication of the genome
  - synthesis of new proteins to make new capsids and cores.
- The details of these processes differ for different types of viruses and are described below for each type.

Viral Genomes

Either DNA or RNA, never both.

DNA viruses can be further divided into

- those that have their genes on a **double-stranded DNA** molecule (**dsDNA**). Example: smallpox
- those that have their genes on a molecule of **single-stranded DNA** (**ssDNA**). Example: Adeno-Associated Virus (AAV)

RNA viruses occur in four distinct groups:

1. Those with a genome that consists of single-stranded **antisense RNA**; that is, RNA that is the complement of the message sense. This is also called **negative-stranded RNA**. Examples: measles, Ebola
2. Those with a genome that consists of single-stranded **sense RNA**; that is, the RNA has message sense (can act as a messenger RNA - mRNA). This is also called **positive-stranded RNA**. Examples: poliovirus
3. Those with a genome made of several pieces of **double-stranded RNA**. Example: reovirus.
4. **Retroviruses**. Their RNA (also single-stranded) is copied by **reverse transcriptase** into a DNA genome within the host cell. Example: HIV-1

DNA Viruses

Genome is a molecule of double-stranded DNA

Examples:

- **smallpox** (variola)
- **vaccinia** (used to immunize against smallpox until the disease was eliminated from the planet)
- **varicella-zoster** (causes chicken pox the first time; shingles the second)
- **herpesviruses**
  - **herpes simplex viruses**
    - **HSV-1 -** usually infects the trigeminal nerves periodically causing "cold sores" on the lips and face
    - **HSV-2 -** usually infects the genitals
  - **KSHV**; causes **Kaposi's sarcoma** in AIDS patients and other people with suppressed immune systems. Also called human herpesvirus 8 (HHV-8).
  - **human cytomegalovirus** (**HCMV**); most of us have it; can cause blindness - even death - in people with suppressed immune systems.
  - **Epstein-Barr virus** (**EBV**); causes **mononucleosis** and has been implicated in the development of Burkitt's lymphoma (a cancer) and Hodgkin's disease. Its genome has been completely sequenced: 172,282 base pairs of DNA encoding 80 genes.
- **adenoviruses**; some 50 different strains infect humans; responsible for some cases of the common "cold". Two strains have been modified to serve as vectors in gene therapy trials.
- **papilloma viruses**; several dozen types infect humans and two of these, HPV-16 and HPV-18, can cause cancer.
- **SV40**; a virus that infects primate cells and causes tumors in rodent cells.
- Some **bacteriophages**
  - **T2** and **T4**; from which much early information about gene structure and expression was learned.
  - **lambda**; a popular vector

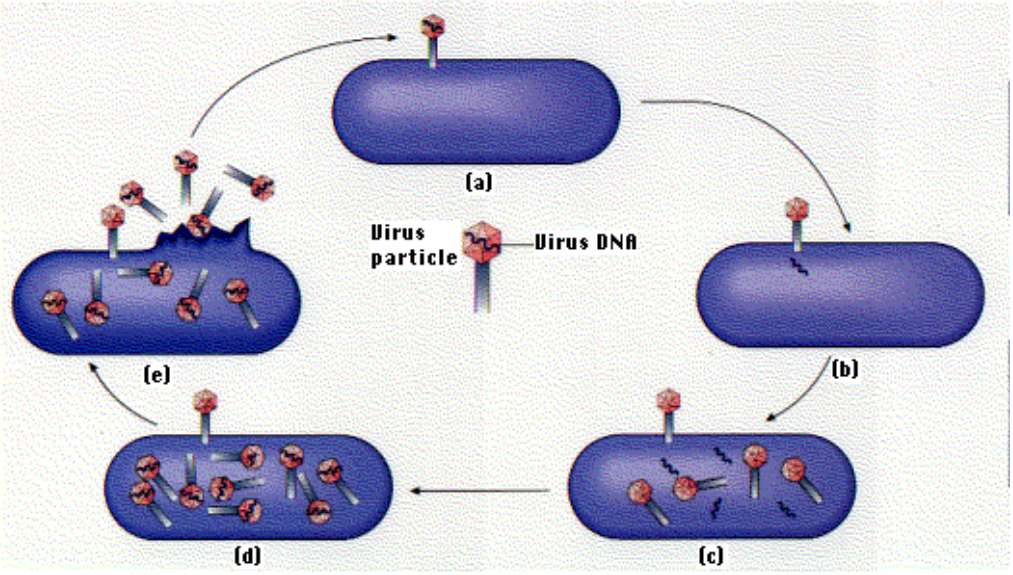


Figure 19.3.1.2 Infective cycle of DNA bacteriophages

The essential elements of the infective cycle of DNA bacteriophages consist of:

- The virions attach to the surface of their host cell **(a)**.
- The proteins of the capsid inject the DNA core into the cell **(b)**.
- Once within the cell, some of the bacteriophage genes (the "early" genes) are transcribed (by the host's RNA polymerase) and translated (by the host's ribosomes, tRNA, etc.) to produce enzymes that will make many copies of the phage DNA and will turn off (even destroy) the host's DNA.
- As fresh copies of phage DNA accumulate, other genes (the "late" genes) are transcribed and translated to form the proteins of the capsid **(c)**.
- The stockpile of DNA cores and capsid proteins are assembled into complete virions **(d)**.
- Another "late" gene is transcribed and translated into molecules of **lysozyme**. The lysozyme attacks the peptidoglycan wall (from the inside, of course).
- Eventually the cell ruptures and releases its content of virions ready to spread the infection to new host cells **(e)**.

Hepatitis B

The genome of hepatitis B ("serum hepatitis") is also **dsDNA**, but its mode of replication is different from the other dsDNA viruses.

- Once inside its host cell (a liver cell), the virion core enters the nucleus.
- The viral DNA is transcribed (by the host's Pol II) into molecules of **mRNA**.
- These enter the cytoplasm where they are translated (again by host ribosomes, etc.) into the various proteins of the virus, including a viral **reverse transcriptase**.
- These components are assembled into new viral cores, and in each
- one molecule of mRNA is reverse transcribed into a single strand of DNA, which then serves as the template for the synthesis of the second strand.

Genome is single-stranded DNA

Examples:

- **φX174** (**phiX174**), another famous bacteriophage (infects *E. coli*) that helped usher in the modern era of molecular genetics. Its single strand of DNA has 5,386 nucleotides and contains 11 protein-encoding genes.
- **Adeno-associated virus** (**AAV**). This virus, which can only grow in cells infected with adenovirus, shows great promise as a safe and effective vector for introducing therapeutic genes into human patients.

RNA Viruses

Negative-stranded RNA viruses:

Genome consists of one or more molecules of single-stranded **"antisense"** RNA.

Examples:

- **measles**
- **mumps**
- **respiratory syncytial virus** (**RSV**), **parainfluenza viruses** (**PIV**), and human **metapneumovirus**. (In the U.S., these close relatives account for hundreds of thousands of hospital visits each year, mostly by children.)
- **rabies**
- **Ebola**
- **influenza**

Method of replication

- In addition to its antisense RNA genome, the core of the virion contains an **RNA replicase**, which is an RNA-dependent RNA polymerase.
- Once released in the host cell, this polymerase makes many **complementary** copies of the genome, which are "sense" and serve as **messenger RNAs**.
- These are translated into the proteins needed to assemble fresh virions, e.g., capsid proteins and RNA polymerase.

Note that this strategy

- provides many copies of mRNA
- depends on the virion having its own RNA replicase (because the host cell does not) (So, naked RNA molecules of these viruses are not infectious — in contrast to the next group: the positive-stranded RNA viruses)

Positive-stranded RNA

Genome is a molecule of single-stranded **"sense"** RNA.

Examples:

- **polioviruses**
- **rhinoviruses** (frequent cause of the common "cold"; 99 different strains are known)
- **noroviruses** (frequent cause of outbreaks of gastrointestinal illness — especially in "closed" settings like cruise ships and nursing homes)
- **coronaviruses** (includes the agent of **S**evere **A**cute **R**espiratory **S**yndrome (**SARS**)
- **rubella** (causes "German" measles)
- **yellow fever** virus
- **West Nile** virus
- **dengue fever** viruses
- **equine encephalitis** viruses
- **hepatitis A** ("infectious hepatitis") and **hepatitis C** viruses
- **tobacco mosaic virus** (TMV)

Method of replication

- The "sense" RNA encodes an **RNA replicase** (an RNA-dependent RNA polymerase) that is translated by the host machinery (ribosomes, etc.) into the enzyme, which catalyzes the synthesis of large numbers of "antisense" **replicative intermediates**.
- These serve as templates for the synthesis of large numbers of **mRNA** molecules that
  - are translated by the host cell machinery into the proteins needed to make fresh virions
  - are incorporated into the new virions.

Double-stranded RNA

Examples:

- reovirus
- several plant viruses

Method of replication

The virus particle contains enzymatic machinery that transcribes each of the dsRNA molecules into a **mRNA** (complete with cap) and exports these into the cytosol of the infected cell.

Retroviruses

These viruses contain a **reverse transcriptase** that copies their RNA genome into DNA.

Examples:

- The Rous sarcoma virus (RSV)
- **HIV-1 and HIV-2**, that cause **AIDS**
- **HTLV-1** and **HTLV-2**. 4–5% of the people infected with HTLV-1 develop adult T-cell leukemia/lymphoma (ATL).

Latent Viruses

Most of the infective cycles described for the various viruses end in the death of the host cell. Bacterial cells literally burst, a process called lysis, and similar infective cycles are called **lytic cycles**.

Lysogeny

In some cases, though, the events of the lytic cycle are not completed. An *E. coli* cell infected by a DNA bacteriophage may resume its normal existence, including reproducing itself.

Where has the virus gone?

It is still there and, in fact, is present in the descendants of the bacterium. That these cells still harbor the virus can be demonstrated by irradiating the cells with ultraviolet rays or treating them with certain chemicals. Such treatment restores the normal lytic cycle. The phage is said to have been "rescued" — hardly the case for its host!

The stable relationship between a bacteriophage and its host is called **lysogeny**. The **viral DNA** actually becomes replicated when the host's DNA is replicated prior to each cell division. During lysogeny, the phage is called a **prophage**.

Transduction

In some cases, the prophage DNA becomes inserted into the chromosome of its host. In fact, when the phage is "rescued", the released virions may contain some host genes as well as their own. When these virions infect new hosts, they insert these bacterial genes into them. This process of genetic transfer, a virus-mediated transformation, is called **transduction**.

What does the prophage do while it is a part of its host genome? It can express certain of its genes. For example, the gene that encodes diphtheria toxin is the property of a prophage in the diphtheria bacillus, not of the bacillus itself.

Some animal viruses can also establish latent infections. **Simian virus 40** (SV40) is a DNA virus that produces

- a **lytic infection** in the kidney cells of the African green monkey (these cells are used to cultivate viruses in the lab)
- but a **latent infection** in the cells of humans, mice, rats, and hamsters. Like lysogeny in bacteria, the SV40 genome becomes incorporated as a **provirus** in the DNA of its host (in chromosome 7 in human cells).

Although a human cell with harboring SV40 shows no outward sign of the provirus, its presence can be detected by:

- the appearance of viral-encoded antigens in the host cell
- the ability of these cells to cause a lytic infection in African green monkey cells when fused with them.

Latent infections may also cause the cell to become cancerous. The cell has become **transformed**. In these cases, the word fulfills both of its biological meanings:

- "transformed" by the incorporation of new DNA
- "transformed" as it becomes cancerous.

In humans,

- **lytic** infections of plasma cells by the **Epstein-Barr virus** (EBV) occur in **mononucleosis**
- **latent** infections of B cells by EBV predispose the person to lymphoma.

while

- **lytic** infections by **human papilloma virus** (**HPV**) cause genital warts;
- **latent** infections by some strains of HPV lead to cervical cancer.

and

- while most $CD4^+$ T cells infected by the retrovirus HIV-1 are killed (causing AIDS),
- HIV-1 integrates as a **provirus** into the DNA of a few memory $CD4^+$ T cells where it can persist for years with the potential of creating active disease in the future.

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19.3B: Influenza

Influenza is a viral infection of the lungs characterized by fever, cough, and severe muscle aches. In the elderly and infirm, it is a major cause of disability and death (often as a result of secondary infection of the lungs by bacteria). Even in the young and healthy, influenza produces a prostrating disease of a few days duration and one not soon forgotten. Influenza is **not** a case of low fever and sniffles that keeps you home in bed for a day nor a gastrointestinal upset ("stomach flu").

Influenza was responsible for the most devastating plague in human history - the "Spanish" flu that swept around the world in 1918 killing 675,000 people in the U.S. and an estimated 20–50 million people worldwide. (A disease that attacks a large fraction of the population in every region of the world is called a **pandemic**.) (It is uncertain where the flu first appeared, but it certainly wasn't in Spain.)

No one at the time even knew what disease agent was causing the pandemic. Not until 1930 (in pigs) and 1933 (in humans) was it established that influenza is caused by a **virus**.

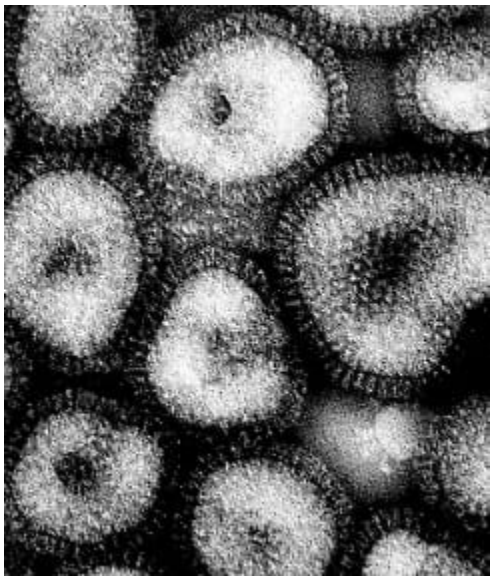


Figure 19.3.2.1 Fluvirions

This electron micrograph (courtesy of Dr. K. G. Murti) shows several influenza virus particles (at a magnification of about 265,000x). The surface projections are molecules of **hemagglutinin** and **neuraminidase** (see below).

There are three types of influenza:

- Common but seldom causes disease symptoms.
- Often causes sporadic outbreaks of illness, especially in residential communities like nursing homes.
- Responsible for regular outbreaks, including the one of 1918. Influenza A viruses also infect domestic animals (pigs, horses, chickens, ducks) and some wild birds.

The Influenza A Virus

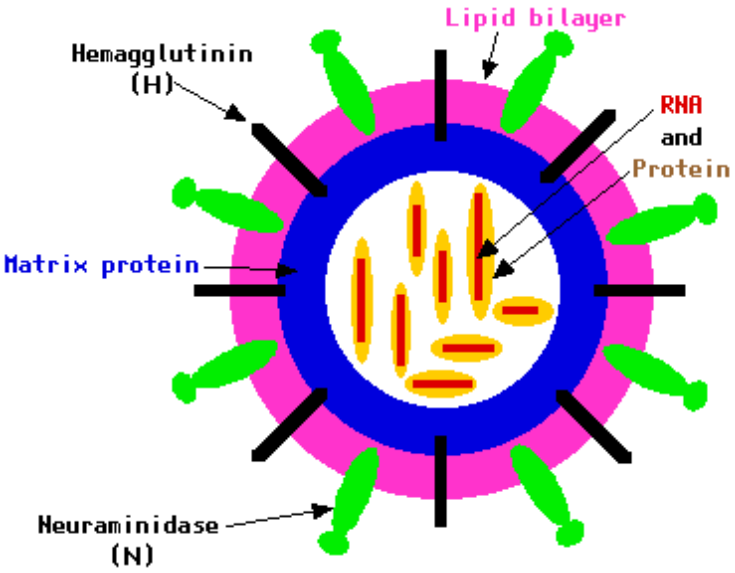


Figure 19.3.2.2 Flu virion

The influenza A virion is

- a globular particle (about 100 nm in diameter)
- sheathed in a **lipid bilayer** (derived from the plasma membrane of its host)
- Studded in the lipid bilayer are three integral membrane proteins
  - some 500 molecules of **hemagglutinin** ("**H**")
  - some 100 molecules of **neuraminidase** ("**N**")
  - the M2 membrane protein (not shown).
- Encased by the lipid bilayer are
  - some 3000 molecules of **matrix protein**
  - **8 pieces or segments** of **RNA**

Each of the 8 RNA molecules is associated with

- many copies of a **nucleoprotein**
- the three subunits of its **RNA polymerase**
- some "non-structural" protein molecules of uncertain function

The Genes of Influenza A

The 8 RNA molecules (the number in brackets is the designated segment number):

- The **HA gene** [4] encodes the **hemagglutinin**. 3 distinct hemagglutinins (**H1**, **H2**, and **H3**) are found in human infections; 15 others have been found in animal flu viruses.
- The **NA gene** [6] encodes the **neuraminidase**. 2 different neuraminidases (**N1** and **N2**) have been found in human viruses; 9 others in other animals.
- The **NP gene** [5] encodes the **nucleoprotein**. Influenza A, B, and C viruses have different nucleoproteins.
- The **M gene** [7] encodes two proteins (using different reading frames of the RNA): a **matrix protein** (M1 — shown in blue) and an ion channel (M2) spanning the lipid bilayer (not shown).
- The **NS gene** [8] encodes two different **non-structural proteins** (also by using different reading frames). These are found in the cytosol of the infected cell but not within the virion itself.
- One RNA molecule (**PA** [3], **PB1** [2], **PB2** [1]) encoding each of the 3 subunits of the **RNA polymerase**. Occasional frameshifts during translation of PA produce a protein that reduces host gene expression.

The Disease

The influenza virus invades cells of the respiratory passages.

- Its hemagglutinin molecules bind to sialic acid residues on the glycoproteins exposed at the surface of the epithelial cells of the host respiratory system.
- The virus is engulfed by receptor mediated endocytosis.
- The drop in pH in the endosome (endocytic vesicle) produces a change in the structure of the viral hemagglutinin enabling it to
- fuse the viral membrane with the vesicle membrane.
- This exposes the contents of the virus to the cytosol.
- The RNA enter the nucleus of the cell where fresh copies are made.
- These return to the cytosol where some serve as messenger RNA (mRNA) molecules to be translated into the proteins of fresh virus particles.
- Fresh virus buds off from the plasma membrane of the cell (aided by the M2 protein) thus
- spreading the infection to new cells.



The result is a viral pneumonia. It usually does not kill the patient (the 1918 pandemic was an exception; some victims died within hours) but does expose the lungs to infection by various bacterial invaders that can be lethal. Before the discovery of the flu virus, the bacterium *Hemophilus influenzae* was so often associated with the disease that it gave it its name.

Pandemics and Antigenic Shift

Three pandemics of influenza have swept the world since the "Spanish" flu of 1918.

- The "Asian" flu pandemic of 1957;
- the "Hong Kong" flu pandemic of 1968;
- the "Swine" flu pandemic that began in April of 2009.

The pandemic of 1957 probably made more people sick than the one of 1918. But the availability of antibiotics to treat the secondary infections that are the usual cause of death resulted in a much lower death rate. The hemagglutinin of the 1918 flu virus was H1, its neuraminidase was N1, so it is designated as an H1N1 "subtype". Here are some others.

Some strains of influenza A

Date	Strain	Subtype	Notes
1918		H1N1	pandemic of "Spanish" flu
1957	A/Singapore/57	H2N2	pandemic of "Asian" flu
1962	A/Japan/62	H2N2	epidemic
1964	A/Taiwan/64	H2N2	epidemic
1968	A/Aichi/68	H3N2	pandemic of "Hong Kong" flu
1976	A/New Jersey/76	H1N1	swine flu in recruits
1977	A/USSR/77	H1N1	"Russian" flu
2009	A/California/09	H1N1	pandemic of "swine" flu [now designated A(H1N1)pdm09]

Until 2009, these data suggest that flu pandemics occur when the virus acquires a **new** hemagglutinin and/or neuraminidase. For this reason, when an H1N1 virus appeared in a few recruits at Fort Dix in New Jersey in 1976, it triggered a massive immunization program (which turned out not to be needed). However, an H1N1 virus appeared the following year (perhaps escaped from a laboratory) causing the "Russian" flu. We now know that this virus was a direct descendant of the 1918 flu. While accumulating mutations that made it less dangerous, it had been infecting humans until it was replaced by the H2N2 "Asian" flu of 1957. Because most people born before the Asian flu pandemic of 1957 had been exposed to the H1N1 viruses circulating before, the Russian flu primarily affected children and young adults. For the same reason, this pattern was also seen in the 2009-10 pandemic of "swine" flu.

Where do the new H or N molecules come from?

Birds appear to be the source. Both the H2 that appeared in 1957 and the H3 that appeared in 1968 came from influenza viruses circulating in birds. The encoding of H and N by separate RNA molecules probably facilitates the **reassortment** of these genes in animals **simultaneously infected by two different subtypes**. For example, H3N1 virus has been recovered from pigs simultaneously infected with swine flu virus (H1N1) and the Hong King virus (H3N2). Probably reassortment can also occur in humans with dual infections.

Epidemics and Antigenic Drift

No antigenic shifts occurred between 1957 ("Asian") and 1968 ("Hong Kong"). So what accounts for the epidemics of 1962 and 1964? **Missense mutations** in the **hemagglutinin** (**H**) **gene**. Flu infections create a strong antibody response. After a pandemic or major epidemic, most people will be immune to the virus strain that caused it. The flu virus has two options:

- wait until a new crop of susceptible young people comes along
- change the epitopes on the hemagglutinin molecule (and, to a lesser degree, the neuraminidase) so that they are no longer recognized by the antibodies circulating in the bodies of previous victims.
    - By 1972, the H3 molecules of the circulating strains differed in 18 amino acids from the original "Hong Kong" strain
    - By 1975, the difference had increased to 29 amino acids.

The gradual accumulation of new epitopes on the H (and N) molecules of flu viruses is called **antigenic drift**. Spontaneous mutations in the H (or N) gene give their owners a selective advantage as the host population becomes increasingly immune to the earlier strains.

Flu Vaccines

Although a case of the flu elicits a strong immune response against the strain that caused it, the speed with which new strains arise by antigenic drift soon leaves one susceptible to a new infection. Immunization with flu vaccines has proved moderately helpful in reducing the size and severity of new epidemics.

Some vaccines incorporate inactivated virus particles; others use the purified hemagglutinin and neuraminidase. Both types incorporate antigens from the three major strains in circulation, currently:

- an A strain of the H1N1 subtype
- an A strain of the H3N2 subtype and
- a B strain.

Because of antigenic drift, the strains used must be changed periodically as new strains emerge that are no longer controlled by people's residual immunity.

The process:

- Chicken eggs are infected with the virus expressing the new H and/or N and **simultaneously infected** with a stock flu virus that grows very well in eggs.
- Genetic reassortment produces some viruses with both the new H and N genes along with the 8 other genes from the stock strain.
- This new virus is then grown in massive amounts and the H and N proteins purified for the new vaccine.

The whole process takes several weeks. A promising way to speed things up is to chemically synthesize the new H and N genes and substitute them for the H and N genes in the stock virus. The new virus can be ready for vaccine production in a few days.

Strains used in vaccines for the flu seasons shown.

Season	H1N1	H3N2	Type B
86–87	A/Chile/83*	A/Mississippi/85	B/Ann Arbor/86

Season	H1N1	H3N2	Type B
* As the 86–87 season got underway, it was found that A/Chile/83 no longer gave protection so A/Taiwan/86 was offered as a second shot late in that season.			
87–88	A/Taiwan/86	A/Leningrad/86	B/Ann Arbor/86
88–89	A/Taiwan/86	A/Sichuan/87	B/Victoria/87
89–90	A/Taiwan/86	A/Shanghai/87	B/Yamagata/88
90–91	A/Taiwan/86	A/Shanghai/89	B/Yamagata/88
91–92	A/Taiwan/86	A/Beijing/89	B/Panama/90
92–93	A/Texas/91	A/Beijing/89	B/Panama/90
93–94	unchanged	unchanged	unchanged
94–95	A/Texas/91	A/Shandong/93	B/Panama/90
95–96	A/Texas/91	A/Johannesburg/94	B/Harbin/94
96–97	A/Texas/91	A/Nanchang/95	B/Harbin/94
97–98	A/Johannesburg/96	A/Nanchang/95	B/Harbin/94
98–99	A/Beijing/95	A/Sydney/97	B/Beijing/93
99–00	A/Beijing/95	A/Sydney/97	B/Yamanashi/98
00–01	A/New Caledonia/99	A/Panama/99	B/Yamanashi/98
01–02	A/New Caledonia/99	A/Panama/99	B/Victoria/00 or similar
02–03	A/New Caledonia/99	A/Moscow/99	B/Hong Kong/2001
03–04	A/New Caledonia/99	A/Moscow/99	B/Hong Kong/2001
04–05	A/New Caledonia/99	A/Fujian/2002	B/Shanghai/2002
05–06	A/New Caledonia/99	A/California/2004	B/Shanghai/2002
06–07	A/New Caledonia/99	A/Wisconsin/2005	B/Malaysia/2004
07–08	A/Solomon Islands/06	A/Wisconsin/2005	B/Malaysia/2004
The B/Malasia component of the vaccine provided no protection at all. So all three components of the 08–09 vaccine were changed as shown on the next line.			

Season	H1N1	H3N2	Type B
08–09	A/Brisbane/2007	A/Brisbane/2007	B/Florida/2006
09–10	A/Brisbane/2007	A/Brisbane/2007	B/Brisbane/2008
Because the 2009–2010 pandemic of the newly-emerged "swine flu" virus drove the "seasonal" H1N1 viruses (e.g., A/Brisbane/2007) to near extinction, the "swine flu" H1N1 – now called A(H1N1)pdm09 – replaced the "seasonal" H1N1 in the 10–11 vaccine.			
10–11	A/California/2009	A/Perth/2009	B/Brisbane/2008
11–12	All three components were unchanged from the previous year		
12–13	A/California/2009	A/Victoria/2011	B/Wisconsin/2010
13–14	A/California/2009	A/Victoria/2011	B/Massachusetts/2012
14–15	A/California/2009	A/Texas/2012	B/Massachusetts/2012
15–16	A/California/2009	A/Switzerland/2013	B/Phuket/2013
16–17	A/California/2009	A/HongKong/2014	B/Brisbane/2008
Several vaccines for the 2016-17 season will be quadrivalent; that is, contain a fourth component B/Phuket/2013.			

FluMist®

On 17 June 2003, the U. S. Food and Drug Administration (FDA) approved FluMist® – a live-virus vaccine that is given as a spray up the nose. The viruses have been weakened so that they do not cause illness, but are able to replicate in the relatively cool tissues of the nasopharynx where they can induce an immune response. Presumably this is tilted towards IgA production, a better defense against infection by inhaled viruses than blood-borne IgG antibodies. In any case, FluMist® induces a more rapid response than inactivated vaccine and there is some evidence that it provides better protection against antigenic drift as well.

All three currently-circulating strains of flu (H1N1, H3N2, and B) are included. As new strains appear, they can be substituted.

At present, this new vaccine (technically known as **LAIV** "Live Attenuated Influenza Vaccine") is only approved for children older than 24 months and adults younger than 50. People with immunodeficiency (e.g., AIDS) should also be cautious about taking it.

Update: For as yet unknown reasons, the nasal spray did not work during the 2015–2016 season, and it is not recommended for the upcoming season.

Flublok®

On 16 January 2013, the U. S. FDA approved an entirely new type of vaccine. Flublok® is made in cell cultures transformed with recombinant DNA encoding the hemagglutinins of the 3 currently circulating flu strains (H1N1, H3N2, and B). The final concentration of antigens is three times that in the current vaccine. Cultures of insect cells are used so there is no problem with possible egg allergies in those receiving the vaccine.

Other weapons against flu

It takes a while for the flu vaccine to build up a protective level of antibodies. What if you neglected to get your flu shot and now an epidemic has arrived?

Amantadine and Rimantadine

These drugs inhibit the M2 matrix protein needed to get viral RNA into the cytosol. They work against A strains only, and resistance to the drugs evolves quickly. By the 2009-2010 flu season, virtually all strains of both H3N2 and H1N1 had developed resistance.

Zanamivir (Relenza®) and Oseltamivir (Tamiflu®)

These drugs block the neuraminidase and thus inhibit the release and spread of fresh virions. Spraying zanamivir into the nose or inhaling it shortens the duration of disease symptoms by one to three days. Unfortunately, by the 2008-2009 flu season, all H1N1 strains circulating in the U.S. had become resistant to Tamiflu.

Antibiotics

Antibiotics are of absolutely no value against the flu virus. However, they are often given to patients to combat the secondary bacterial infections that occur and that are usually the main cause of serious illness and death.

Why so few drugs?

The mechanisms by which amantadine and zanamivir work provide a clue. There are far fewer anti-viral drugs than antibacterial drugs because so much of the virus life cycle is dependent on the machinery of its host. There are many agents that could kill off the virus, but they would kill off host cell as well. So the goal is to find drugs that target molecular machinery unique to the virus. The more we learn about these molecular details, the better the chance for developing a successful new drug.

The "Spanish" Flu

Jeffery Taubenberger and his colleagues have sequenced the genes of the influenza virus that had been recovered from

- preserved lung tissue of a U.S. soldier who died from influenza in 1918
- lung tissue from a flu victim whose body had remained frozen in the permafrost of Alaska since she died in 1918

But even with all of its genes now completely sequenced, why the 1918 strain was so deadly is not fully understood. But deadly it is. They have even been able to replace the 8 genes of a laboratory strain of flu virus with all 8 genes of the 1918 strain (using strict biosafety containment procedures!). The resulting virus kills mice faster than any other human flu virus tested. (Reported in the 7 October 2005 issue of **Science**.)

The Swine Flu of 2009

A new H1N1 flu began infecting humans in North America in April 2009 and has now spread throughout much of the world. Sequencing its genome revealed a novel virus - now called A(H1N1)pdm09 - that contained genes previously found in four different strains of swine flu:

- an **HA** gene (H1) derived from the swine flu of 1930 (and closely-related to the H1 of the great 1918 "Spanish" flu pandemic) along with an **NP** and **NS** gene from that virus;
- an **NA** gene (N1) from a virus that had been circulating in the pigs of Europe and Asia since 1979 along with the **M** gene from that virus;
- a **PA** and **PB2** gene that entered pigs from birds around 1998;
- a **PB1** gene that passed from birds to humans around 1968 and from us to pigs around 1998.

Why this remarkable assortment of genes has enabled he virus to jump so successfully from pigs to humans remains to be determined.

The amino acid sequence around the critical epitopes of its H1 molecules closely resemble those found in the resurrected 1918 flu virus. This would explain why

- Antibodies from elderly survivors of the 1918 pandemic neutralize the new swine flu virus.
- Antibodies (raised in mice) to the new swine flu virus neutralize the resurrected 1918 flu virus.
- The recent pandemic caused serious illness and death mostly in young adults and least in children and the elderly. As for the elderly, this contrast to the usual pattern arose because people over 65, even if not old enough to have been exposed to the 1918 virus, had been exposed to H1 viruses that until 1957 had only drifted from the original 1918 virus, and thus they had developed partial immunity. The antibodies in young adults were specific for seasonal flu strains circulating since 1957. These were unable to protect them against the 2009 virus but may have formed damaging immune complexes with them. Youngsters had no anti-flu antibodies and did not form such immune complexes.

"Bird Flu"

Many influenza A viruses are found in birds, both domestic and wild. Most of these cause little or no illness in these hosts. However, some of their genes can enter viruses able to infect domestic animals, as was the case for the PA and PB2 genes of the swine flu of 2009 (above).

On several occasions, bird flu viruses have also infected humans, often with alarmingly-high fatality rates. In 2003, human cases of an H7N7 bird flu virus infection occurred in the Netherlands, and in the same year an H5N1 bird virus caused human cases in large areas of Asia. Most of the human cases seemed to have been acquired from contact with infected birds rather than from human-to-human transmission.

And now in 2013, a new bird flu virus, H7N9, has appeared in humans in China. By the end of the summer of 2013, it had caused 135 observed cases (no one knows yet whether there may also be infected people who are not sick enough to show up at hospitals). 45 of the observed cases were fatal. The victims appear to have been infected through contact with infected poultry with little or no evidence of human-to-human transmission.

As a glance at the tables above will show, humans have had long experience with infections and vaccines by both H1 and H3 flu viruses. But the human population has absolutely no immunity against any H7 viruses. If this virus develops the capability to spread efficiently from human to human, it could lead to another worldwide pandemic.

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19.3C: φX174

φX174 (phiX174) is a virus that infects the bacterium *E. coli*. Hence φX174 is a **bacteriophage**. Each complete infectious particle (virion) of φX174 consists of a protein coat which envelopes a core that contains both protein and DNA. The coat of the virus contains 60 molecules each of two proteins (F and G) and 12 molecules of another protein (H). The core of the virion contains one molecule of DNA and 60 copies of a fourth protein, the J protein. The DNA molecule is single-stranded (ssDNA) and is in the form of a closed circle. It contains 5386 nucleotides. This tiny genome was the first DNA genome ever to be sequenced (by Fred Sanger in 1976).

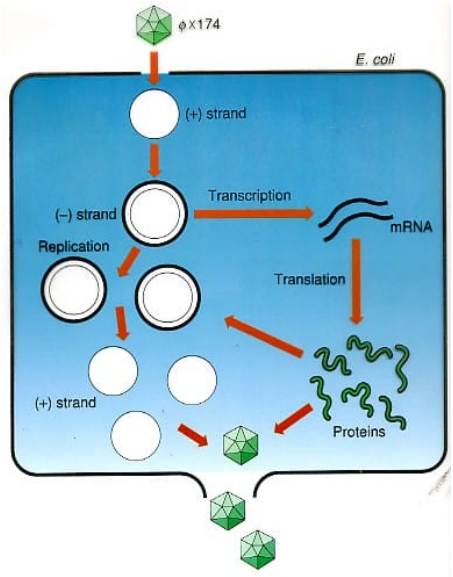


Figure 19.3.3.1 PhiX

When φX174 attaches to its host, its ssDNA molecule is inserted into the cell. Here the DNA strand (+) serves as the template for the synthesis of a complementary (–) strand. The two strands form a double helix which then replicates itself several times. The minus strands of these DNA molecules then serve as templates for the synthesis of:

- mRNA molecules.
- some 200 complementary (+) strands of DNA, each of which will later be packaged into the core of a new virion.

The protein-synthesizing machinery of the host cell translates the viral mRNA molecules into 11 different kinds of proteins. Four of these are the four (F, G, H, and J) that will be incorporated into new virions. As for the other 7 proteins

- A, A*, and C play roles in the replication of viral DNA
- B and D assist in the assembly of the virion proteins into new virions
- E lyses the host cell so the newly-synthesized virions can escape
- K boosts virion production

but none of these proteins become part of the virion.

Protein	Number of Amino Acids
A	513
A*	341
B	120
C	86
D	152
E	91
F	427
G	175
H	328
J	38
K	56
Total	1986

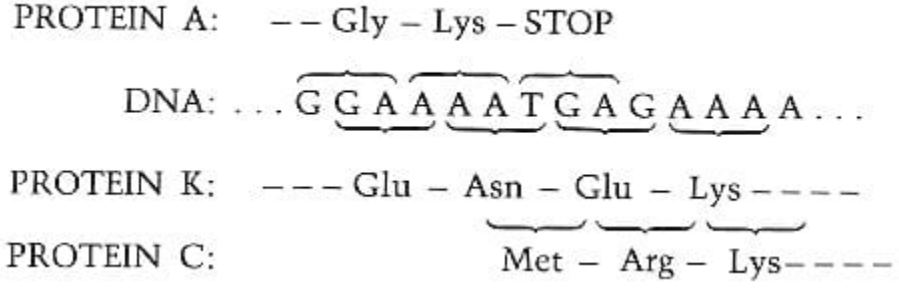
The 11 proteins encoded by φX174 DNA range in size from the A protein, which contains 513 amino acids, to the J protein, which contains only 38. The 11 proteins together contain a total of 1986 amino acids (the A* protein is simply a shortened version of the A protein). This raises a question. With 3 nucleotides needed to specify one amino acid, φX174 would need 5958 nucleotides to encode 1986 amino acids (5958/3 = 1986). But its DNA molecule contains 5386 nucleotides, only enough to encode 1795 amino acids. Furthermore, it turns out that 217 of the nucleotides do not encode anything, although some of them provide control signals. So there are only 5169 coding nucleotides, and we would expect them to be able to encode only 1723 amino acids. How does φX174 dictate the assembly of the remaining amino acids?

Overlapping Genes

It does so by using some stretches of nucleotides to encode two different sequences of amino acids. The principle is really quite simple. It involves reading the codons it two different "reading frames", that is, grouping the nucleotides in shifted clusters of three.

For example, the sequence
. . . GAGCCGCAACTTC . . .
can be read in three different reading frames:
. . . GAG CCG CAA CTT C . . . which encodes . . Glu-Pro-Gln-Leu . .
or
. . . G AGC CGC AAC TTC . . . which encodes . . Ser-Arg-Asn-Phe . .
or
. . . GA GCC GCA ACT TC . . . which encodes . . Ala-Ala-Thr . .

φX174 actually uses two of these and, as you can see, each encodes a totally different sequence of amino acids.



There is even one spot where a single nucleotide (A) participates in three different codons:

- It is the third nucleotide in the codon (AAA) for the final amino acid (Lys) in protein A;
- the middle nucleotide in the codon AAT, which encodes Asn in the K protein; and
- the first nucleotide in ATG, the codon that places methionine (Met) at the start position of protein C.

Why overlapping genes? **φX174** is one of the tiniest viruses. Its use of overlapping genes enables it to increase the amount of information it can store in a given amount of DNA. Not only was the φX174 genome the first to be sequenced, it was also the first to be chemically synthesized in the laboratory. When introduced into *E. coli*, this synthetic molecule was fully infectious able to produce intact viruses.

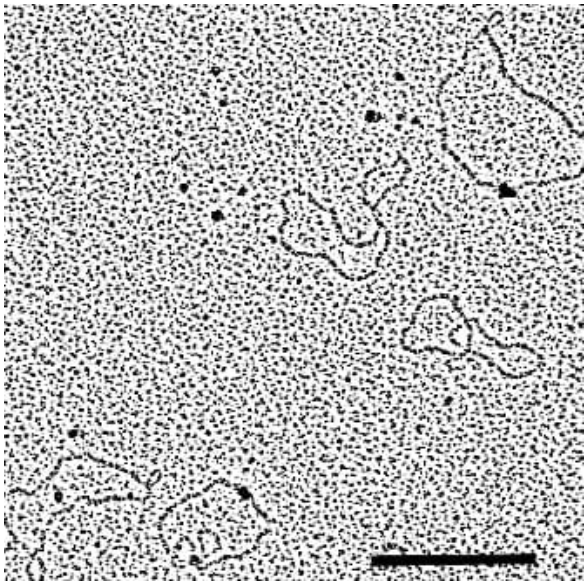


Figure 19.3.3.2 PhiX174

Above is an electron micrograph of the double-stranded φX174 DNA extracted from infected *E. coli* cells. The bar represents 0.5 µm. (Courtesy of David T. Denhardt.)

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19.3D: Smallpox

The threat of terrorism has raised the spectre of the use of biological agents as weapons. One of the possible agents is the **variola virus**, the cause of smallpox. On October 26, 1977, Ali Maow Maalin came down with smallpox in the town of Merka in Somalia. Within a few weeks he was fully recovered. Since that time, not a case of smallpox (except as a result of one laboratory accident) has been discovered anywhere in the world. By May of 1980, the World Health Organization (WHO) felt that it could confidently announce that smallpox had been completely eradicated. The WHO also asked that all countries with any stocks of variola virus in their laboratories either destroy them or transfer them to one of two secure laboratories (at the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia or a state lab in Koltsovo in Russia). Although 74 countries did so, the fear remains that some countries may have retained stocks of the virus. Even before the complete eradication of smallpox, routine vaccination against the disease was halted in most Western countries. So today anyone under 30 years of age is fully susceptible and even those older may have lost protection against the disease.

A Little History

Smallpox certainly qualified as one of the greatest scourges of humanity. It regularly killed 25% and sometimes as many as 50% of its victims. Introduced into Europe around the sixth century A.D., smallpox rivaled plague in its ability to decimate entire populations. Introduced into the New World in the sixteenth century, smallpox devastated the native populations and played a far greater role than weaponry in the Spanish Conquest.

How was such a pestilence eradicated? Four factors were decisive:

1. The variola virus, which causes the disease, attacks only humans; no animal reservoirs have been found (as they have for the yellow fever virus, the rabies virus, and the plague bacillus).
2. If the victim recovers, the virus is completely eliminated from the body. There are no smallpox "carriers" as there are for such diseases as typhoid fever and malaria.
3. An effective vaccine was available. The vaccine could quickly establish a strong (and reasonably long-lasting) immunity. Thus the chain of contagion could be quickly broken by vaccinating all possible contacts associated with a new case.
4. The WHO and the countries involved provided personnel, money, and the determination to do the job. An effective vaccine had, as we shall see, been available since 1796 and had already rendered many parts of the world free of the disease during the first half of the 20th century. But still the disease smoldered in Asia, Indonesia, Brazil, and Africa. Only a heroic public health effort — a campaign that began in 1967 — finally eliminated it worldwide.

Variolation

The first effective attempts to cope with smallpox were made in some of the same regions - Asia, India, Africa - that were the last to be freed of the disease. The technique was deliberately to inoculate susceptible individuals (i.e., those with no pockmarks to indicate that they had survived an earlier epidemic) with material taken from the pustules of people with a mild case of the disease. This practice, called variolation, induced an active case in the recipient, but usually the case was less severe than if the disease had been contracted in the normal way (by inhalation as it turned out).

Variolation was introduced into England and the American colonies early in the 18th century. The practice was often accompanied by violent controversy. It was not entirely safe. The variolated person often became quite ill and the mortality rate, although only a fraction of that for people who contracted the disease in the normal way, was nonetheless appreciable. But far more significant in terms of public acceptance was the fact that variolated people were fully contagious to others during the period of their brief, hopefully mild, illness. Thus a family electing variolation could start a fresh smallpox epidemic. Nonetheless, the practice gradually gained favor until it was replaced by vaccination. This table (from J. B. Blake, *Public Health in the Town of Boston, 1630-1832*. Harvard University Press, 1959) shows the effect of variolation on the death rate from smallpox during three epidemics in Boston.

Year	1721	1764	1792
Population	10,700	15,500	19,300
Natural Smallpox			

Cases	5,759	699	232
Deaths	842	124	69
Deaths/1000 cases	146	177	298
Smallpox Caused by Variolation			
Cases	130	4,977	9,152
Deaths	2	46	179
Deaths/1000 cases	15	9	20

Vaccination

Edward Jenner was a Gloucestershire physician who introduced the practice that led to the elimination of smallpox. Jenner's success was grounded on two observations:

1. The regional folk belief that if a milkmaid had ever contracted **cowpox**, she would never contract smallpox.
2. The inability to variolate successfully those who had an earlier case of **cowpox**. Cowpox is a disease that produces pustules on the teats and udders of cows. Persons in close contact with cows frequently contracted the disease and suffered a mild and transient infection.

Jenner systematically exploited these observations.

- First he deliberately induced cowpox in his human subjects by inoculating them with material from cowpox pustules.
- Then he showed that these individuals could NOT be **variolated**.

Jenner's procedure, which we call **vaccination**, (L. *vacca*, cow) quickly replaced variolation as a public health measure because:

- Any reaction it induced was far milder than the disease induced by variolation.
- The vaccinated subject was not contagious to others.

Jenner's was the first safe and successful attempt to artificially induce an active immunity. Many successful attempts have followed since Jenner's day, but the principles that guided him are still followed:

- Develop a harmless (or as harmless as possible) preparation that will, upon introduction into the body,
- induce a response that will protect the individual from a **harmful** pathogen.

Because of Jenner's priority and his success, the term **vaccine** is used today for all such preparations. The administration of a vaccine is called **immunization**. The virus used in today's smallpox vaccine is called **vaccinia virus**. Possibly it is a relative of cowpox virus, but when the switch occurred is lost in the obscurity of the years since Jenner's day.

What's Next?

Jenner himself was so confident of the efficacy of vaccination that he wrote:

> **"The annihilation of smallpox must be the final result of this practice".**

In 1980, his prediction seemed to have been fulfilled. Today we are not so confident. What should we do now? Return to universal vaccination or use the vaccine only for emergency and medical people who might be exposed as they responded to a terrorist attack and those people in a "ring" around any person who comes down with the disease.

The argument against universal vaccination is that present vaccines are not 100% safe. There is a small, but definite, risk of serious complications from the vaccine itself, especially in people who have an immunodeficiency (e.g., from AIDS or taking immunosuppressant drugs). Such problems can be avoided by not giving the vaccine to people at risk. It can also be avoided by having **Vaccinia Immune Globulin** (**VIG**) available to treat any cases of a bad response to the vaccine.

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19.3E: Retroviruses

The genome of retroviruses consists of **RNA** not DNA. **HIV-1** and HIV-2, the agents that cause **AIDS**, are retroviruses. In February 1997 it was reported that pig cells contain a retrovirus capable of infecting human cells (at least, in vitro). This is troublesome because of the efforts that are being made to transplant pig tissue into humans (e.g., fetal pig cells into the brains of patients with Parkinson's disease). Transplant recipients must have their immune systems suppressed if the transplant is to avoid rejection. Could immunosuppressed patients be at risk from the retroviruses present in the transplanted cells? The probability that the original hosts for HIV-1 and HIV-2 were some other primate suggests that retroviruses can move from one species to another.

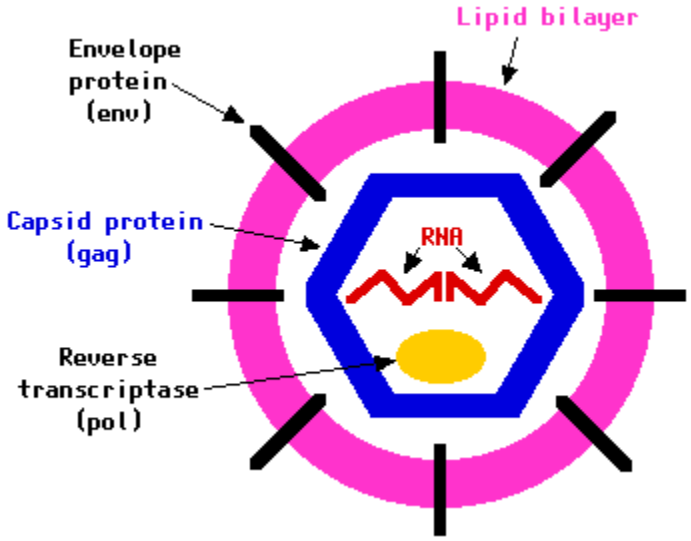


Figure 19.3.5.1 Retrovirus

A typical, "minimal" retrovirus consists of:

- an outer **envelope** which was derived from the plasma membrane of its host
- many copies of an **envelope protein** embedded in the lipid bilayer of its envelope
- a **capsid**; a protein shell containing
- two molecules of **RNA**
- molecules of the enzyme **reverse transcriptase**

Reverse transcriptase is a DNA polymerase that uses RNA as its template. Thus it is able to make genetic information flow in the reverse (RNA ->DNA) of its normal direction
(DNA -> RNA).

Infection of a host cell requires that the cell have a surface protein that can serve as a **receptor for the envelope protein** of the retrovirus. The envelope protein of HIV-1 binds to **CD4** molecules (this property enables the virus to invade $CD4^+$ T cells and certain other cells that express CD4) and they bind to CCR5 (CC chemokine Receptor 5) - found on Th1 cells and macrophages. All the proteins in the virus particle are encoded by its own genes.

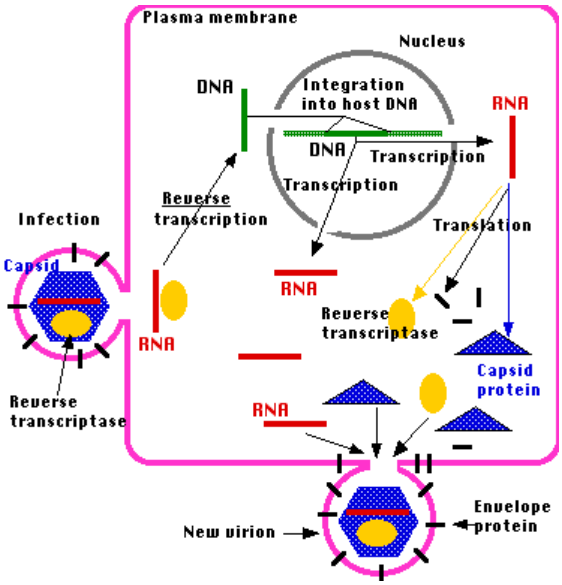


Figure 19.3.5.2 Retrovirus lifecycle

When a retrovirus infects a cell

- Its molecules of reverse transcriptase are carried into the cell attached to the viral RNA molecules.
- The reverse transcriptase synthesizes DNA copies of the RNA.
- These enter the nucleus and are inserted into the DNA of the host.
- These inserts are **transcribed** by the host's enzymes into fresh **RNA** molecules which re-enter the cytosol where
  - some are **translated** by host ribosomes
    - the **gag** gene is translated into molecules of the **capsid protein**
    - the **pol** gene is transcribed into molecules of **reverse transcriptase**
    - the **env** gene is translated into molecules of the **envelope protein**
  - other RNA molecules become incorporated into fresh virus particles

The genome of retroviruses is flanked at each end by repeated sequences ("**R**") that enable the DNA copy of the genome to be inserted into the DNA of the host and act as enhancers, causing the host nucleus to transcribe the DNA copies of the retroviral genome at a rapid rate.



The retroviral genome also contains a **packaging signal** sequence ("**P**") which is needed for the newly-synthesized RNA molecules to be incorporated in fresh virus particles. Most retroviruses also contain one or more additional genes. Some of these represent RNA copies of genes that earlier were picked up from their eukaryotic host. Several cancers in animals are caused by retroviruses that have, at some earlier time, picked up a proto-oncogene from their mammalian host and converted it into an **oncogene**.

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UNIT 20: GENERAL SCIENCE

20.1: Epidemiology
20.2: Types of Clinical Studies
20.3: Scientific Methods
20.4: Scientific Papers
20.5: Statistical Methods
20.6: Drugs

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20.1: Epidemiology

A statistical correlation between two phenomena is simply that. It does not prove that one phenomenon caused the other. Just because **A** is often associated with **B**, does not prove that A **causes** B. Example: the incidence of cirrhosis of the liver is associated with cigarette smoking. Does smoking **cause** cirrhosis? Probably not. Excessive consumption of alcohol is a more likely cause. However, as heavy drinkers tend to be heavy smokers, the statistical association is there, but in this case is probably a confounding variable. How can one establish that A causes B? In the laboratory you could set up a controlled experiment treating one group of animals with A and having a second **control group** without A but otherwise treated the same (thus avoiding confounding variables). Such experimentation is rarely possible (or ethical) in humans so we must turn to the methods and criteria of epidemiology.

John Snow - the First Epidemiologist

During an outbreak of cholera in London in 1854, John Snow plotted on a map the location of all the cases he learned of. Water in that part of London was pumped from wells located in the various neighborhoods. Snow's map revealed a close association between the density of cholera cases and a single well located on Broad Street. The Broad Street well is marked with an X (within the red circle) in the above figure. Removing the pump handle of the Broad Street well put an end to the epidemic. This despite the fact that the infectious agent that causes cholera was not clearly recognized until 1905.



Figure 20.1.1: John Snow's map showing cholera deaths in London in 1854 (courtesy of The Geographical Journal)

Although an association between two phenomena is no more than that, one can apply several criteria to gauge the **strength of the association**, and if it is strong, infer that one phenomenon **causes** the other.

The five criteria to gauge the strength of the association

- a high relative risk
- consistency
- a graded response to a graded dose
- a temporal relationship
- a plausible mechanism

Cigarette Smoking: A Case Study

In Table 20.1.1, the quotient of **observed deaths** divided by **expected deaths** (those in the control group) gives the **relative death rate**. This value is a measure of **risk**. Although smoking is associated with many more cases of heart disease than of lung cancer, lung cancer is the disease with the highest relative risk for smokers. The relative death rate from lung cancer is over 10 times greater in smokers than in non-smokers. This is strong evidence that smoking causes lung cancer. Cigarette smoking is estimated to be directly responsible for 80–90% of all lung cancer deaths (which totalled 160,390 in the U.S. in 2007).

This table gives the number of deaths from various causes in a prospective study of cigarette smokers ("observed deaths") compared with the number to be expected among nonsmokers of the same ages ("expected deaths"). The differences between the two represent "excess deaths". The contribution of each disease to the total of excess deaths is given as the "percentage of excess". Note that coronary artery disease accounts for one half of the excess deaths in the smoking group.

Table 20.1.1: Observed deaths, expected deaths, and relative death rates.

Cause of Death	Observed Deaths	Expected Deaths	Excess Deaths	Percentage of Excess	Relative Death Rate
Total deaths (all causes)	7316	4651	2665	100.0	1.57
Coronary artery disease	3361	1973	1388	52.1	1.70
Other heart disease	503	425	78	2.9	1.18
Cerebrovascular lesions	556	428	128	4.8	1.30
Aneurysm & Buerger's disease	86	29	57	2.1	2.97
Other circulatory diseases	87	68	19	0.7	1.28
Lung cancer	397	37	360	13.5	10.73
Cancer of mouth, larynx, or esophagus	91	18	73	2.7	5.06
Cancer of the bladder	70	35	35	1.3	2.00
Other cancers	902	651	251	9.4	1.39
Gastric & duodenal ulcer	100	25	75	2.8	4.00
Cirrhosis of the liver	83	43	40	1.5	1.93
Pulmonary disease (except cancer)	231	81	150	5.6	2.85
All other diseases	486	453	33	1.2	1.07
Accident, violence, suicide	363	385	-22	-0.8	0.94

Dividing the number of observed deaths by the number of expected deaths gives the "relative death rate" for each disease. This shows that smokers die of lung cancer **10 times as often** (**10.73**, above) as do nonsmokers, which is a very high **relative risk**. However, in both groups lung cancer is rarer than coronary artery disease. (Data from E. C. Hammond and D. Dorn, 1966.)

Consistency

Our confidence that A causes B is strengthened when different studies using different populations all show the same association. The earliest studies of smoking were **retrospective**; that is, after a disease was diagnosed, the patient's smoking habits were determined. Later studies were **prospective**. A prospective study selects a population in good health and meeting any other desired criteria (smoking habits in this case) and follows it over a period of years to see what happens to its members.

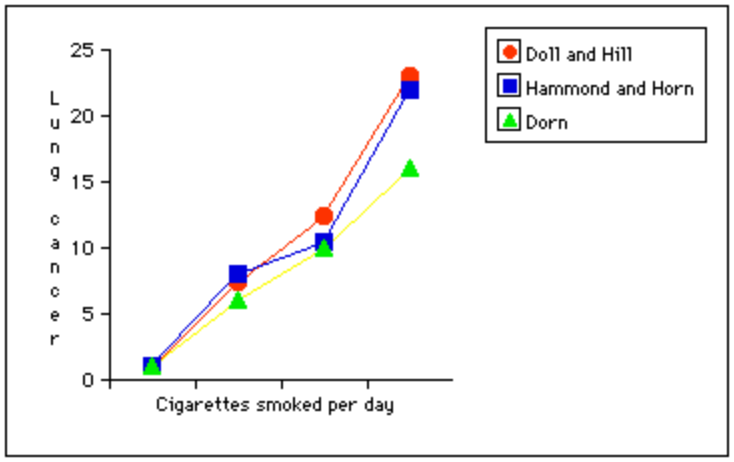


Figure 20.1.2: Relationship between smoking and death

This graph shows essentially the same relationship between smoking and deaths from lung cancer in three different groups (totalling over a million people) studied prospectively. Doll and Hill studied a group of British physicians. Dorn followed the health of a group of U.S. veterans. Horn studied 187,783 U.S. male volunteers. In each case the relative death rates are graphed as a function of number of cigarettes smoked each day (from zero at the left to over a pack at the right).

A Graded Response to a Graded Dose

All three studies graphed above show that the relative death rate from lung cancer increased with an increase in the average number of cigarettes smoked each day. One goal of picking different groups to study is to avoid **confounding variables**. If, for example, all the groups studied lived in cities, it would be difficult to distinguish between the effects of smoking and the effects of general air pollution.

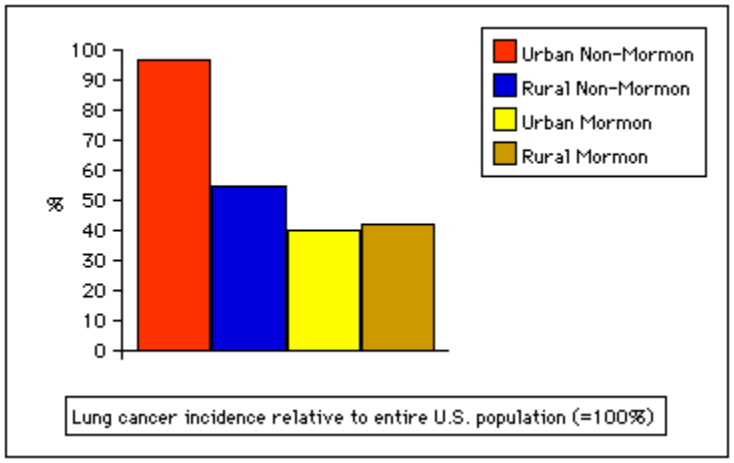


Figure 20.1.3: Utah smokers

This graph compares the incidence of lung cancer among male Mormons and non-Mormons living in urban and rural areas of Utah. Male **non**-Mormons living in the city have a higher risk of developing lung cancer than those living in the country. Is this because of smoking or because of the pollution of urban air? It appears to be the former because Mormons show no such city vs. country difference, and cigarette smoking is prohibited for Mormons. Studies like these help to eliminate the effect of confounding variables. Probably less than 5% of lung cancer is caused by breathing polluted city air.

Temporal Relationship

If A causes B, then exposure to A must have preceded the onset of B. Establishing cause-effect relationships for possible carcinogens has been particularly difficult because for cancers, the latency period between exposure and illness is often many years. Nonetheless, data such as those shown in this graph, provide another strong link in the case against cigarettes.

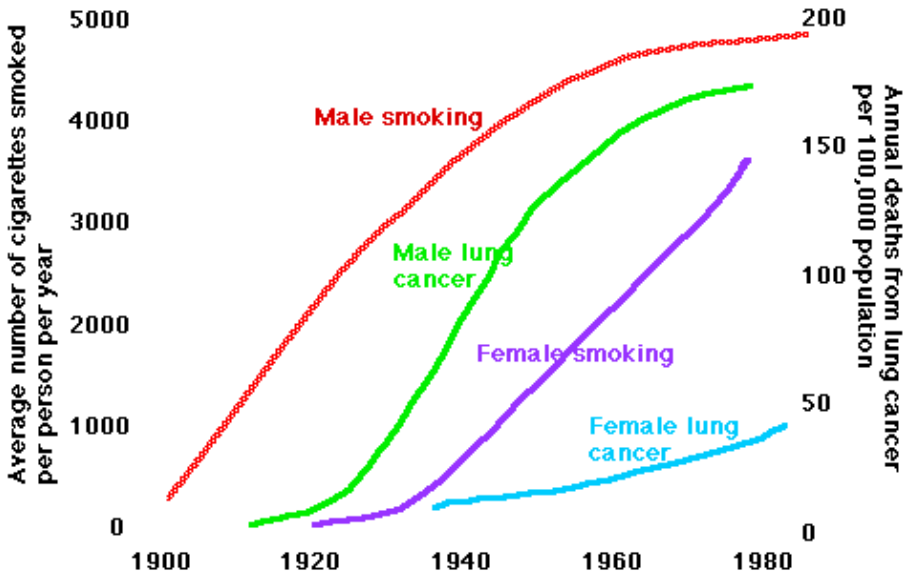


Figure 20.1.4: Cause - effect relationship for possible carcinogens

In recent decades, sales of cigarettes in the U.S. have dropped, both on a per capita basis and in absolute numbers. Whereas half of adult males smoked in the mid-sixties, less than a third do today. This change has already caused the rate of lung cancer in males to level off. Unfortunately, the rate is still rising for women (and in 1987 surpassed breast cancer as the leading cause of cancer deaths in U.S. women).

Plausible Mechanism

Over 40 different chemicals found in cigarette smoke cause an increase in cancer when given over several years to laboratory rats.

So how strong is the case against cigarettes?

Defenders of the tobacco industry frequently claim that no one has **proved** that cigarette smoking causes lung cancer. In one sense they are right. Proof from epidemiology differs from proof in a laboratory experiment. What we have seen here is that the more closely we can meet the several criteria linking A and B, the more confident we can be that A causes B.

Few epidemiological studies have met these criteria better than those studying the statistical relationship between smoking and health. Smoking is probably the greatest single cause of preventable illness in the United States.

Rules to live by

Hardly a week goes by these days without a report in the press and on TV of another link between an environmental agent and human disease. Does living near nuclear power stations increase one's risk of cancer? living near electric power lines? Does a diet rich in saturated fats predispose U.S. males (but, for some reason, not French males) to early death from coronary artery disease?

I hope that your ability to interpret the avalanche of reported associations - and any adjustments that you make in your life as a result - will benefit from your applying to such reports the five criteria outlined here.

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20.2: Types of Clinical Studies

Researchers in human (and veterinary) medicine are always on the lookout for new drugs, medical procedures, and life-style changes that will improve their ability to bring better health to patients. For each one, they must establish whether it truly represents an improvement from what was used before.

Retrospective Studies

In a typical retrospective study, the health profiles of the subjects in a particular "case" group (e.g. smokers) are compared with those in a **control group** that has been selected to be as similar as possible to the "case" group (similar spread of ages, sex, etc.). Retrospective studies are also called "**case-control**" studies. Retrospective studies run the risk of investigator bias; that is, the investigator picks subjects that are most likely to show the effect that prompted the study in the first place.

Prospective Studies

A prospective study selects a population in good health and meeting any other desired criteria (e.g., smoking habits) and follows it over a period of years to see what happens to its members. Prospective studies are also known as "**cohort studies**".

Clinical Trials

Where it is possible to do them, clinical trials represent the "gold standard" for evaluation. In performing a clinical trial (e.g. of a new, and possibly better, drug), the investigator

- assembles a group of subjects picked to be as similar as possible in their characteristics (to avoid "confounding variables")
- assigns them **randomly** to the experimental group and a "control" group
- performs the experiment on the first group. It is best to do this in a "double-blind" fashion; that is neither the subject nor the experimenterknows who is getting the treatment and who is not.

Keeping

- the subjects in the dark avoids the placebo effect; a response (often quite powerful) that is not due to the treatment but to the expectations of the subject;
- the experimenter in the dark avoids bias in the evaluation of the results. So, for example, a microscopist examining a slide of tissue should not know whether it came from an experimental subject or a control.

Analyzing the Data

The data acquired in any type of clinical study must be evaluated to see if any effect seen is significant.

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20.3: Scientific Methods

There is nothing mysterious or even particularly unusual about the things that scientists do. There are many ways to work on scientific problems. They all require common sense. Beyond that, they all display certain features that are especially - but not uniquely - characteristic of science.

For example:

- **Skepticism**. Good scientists use highly-critical standards in the judging of evidence. They approach data, claims, and theories (ideally, even their own!) with healthy doses of skepticism.
- **Tolerance of uncertainty**. Scientists often work for years - sometimes for an entire career - trying to understand one scientific problem. This often involves finding facts that, for a time, fail to fit into any coherent pattern and that even may support mutually contradictory explanations.

Sometimes, as one listens to scientists vigorously defending their views, their confidence seems absolute. But deep in their hearts, they know that their views are based on probabilities and that a new piece of evidence may turn up at any time and force a major shift in their views.

- Although they certainly have no monopoly on hard work, their willingness to work long hours and years pursuing a problem is the mark of all good scientists. For science is hard work.
- Before undergoing the frustrations - tempered by occasional joys - of wresting more secrets from nature, you must learn the foundations on which your subject is based.

Although scientific methods are as varied as science itself, there is a pattern to the way that scientists go about their work.

Scientific advances begin with observations.

- A census of the members of a species in some habitat is an observation.
- The readings on the display of a laboratory instrument are observations.

But science is more than a catalog of facts. The goal of science is to find **an explanation for why the facts are as they are**. Such an explanation is a **hypothesis**.

Testing Hypotheses

A good hypothesis meets several standards:

1. It should provide an adequate explanation of the observed facts.
2. If two or more hypotheses meet this standard, the simpler one is preferred.
3. It should be able to **predict** new facts.

So if a generalization is valid, then certain specific consequences can be **deduced** from it. One of the most exciting events in science is to predict the results of an experiment not yet performed if the hypothesis is valid and then to perform the experiment.

The Null Hypothesis

Experimental biology often involves setting up an experimental treatment and — at the same time — a **control**. Then one compares the results of the experimental treatment with the results in the controls. If there is a difference, what is the probability that it is due to chance alone; that is, the experimental treatment really had no effect?

The hypothesis that the experimental treatment had no effect is called the null hypothesis. Most workers feel that if the probability (designated p) of the observed difference is less than 1 in 20 ($p = <0.05$), then the null hypothesis is disproved and the observed difference is **significant**. But significance is not proof. In fact, hypotheses can never be proven to be absolutely "true" is the sense that a theorem in geometry can. The most we can say is that there is a high probability that the hypothesis provides a valid explanation of the phenomenon being studied.

Hypotheses that are supported by many observations come to be called **theories**. So, in contrast to some areas of human thought, science can never prove that a theory is "true". But it can show that a theory is false. Lest the tentative nature of science cause you to lose confidence in it, think of what science has produced. The many achievements of scientific methods, despite the absence of absolute certainty, has been well-expressed in the sonnet "Paradox" by the late mathematician Clarence Wylie, Jr.

> Not truth, nor certainty. These I foreswore
>
> In my novitiate, as young men called
>
> To holy orders must abjure the world.
>
> "If..., then...," this only I assert;
>
> And my successes are but pretty chains
>
> Linking twin doubts, for it is vain to ask
>
> If what I postulate be justified,
>
> Or what I prove possess the stamp of fact.
>
> Yet bridges stand, and men no longer crawl
>
> In two dimensions. And such triumphs stem
>
> In no small measure from the power of this game,
>
> Played with the thrice-attenuated shades
>
> Of things, has over their originals.
>
> How frail the wand, but how profound the spell!

Reproducibility of Scientific Work

The single feature that is most characteristic of science is its reproducibility. If scientists cannot duplicate their first results, they are forced to conclude that these were invalid. This problem occurs often. Its cause is usually some unrecognized, and hence uncontrolled, factor in the experiment (e.g., unrecognized variation in the properties of different batches of the materials used in the experiment). With luck, the inability to reproduce experiments will be discovered by the same scientists who did the first experiments. This is why scientists generally repeat their experiments several times before reporting them in a scientific paper.

On other occasions, workers in another laboratory fail to secure the same results when they repeat experiments that have been published or, more often, perform experiments designed to carry the study into new areas, but these fail because of a flaw in the original experiments. When this happens, all the parties concerned should get together to see if they can find out why their results differ.

- Often it is simply a matter of not using precisely the same materials and methods.
- Sometimes, however, a serious flaw may be discovered in the design and/or execution of the original experiments.
- And sometimes it proves impossible to find out why experiments that once seemed to work no longer do so.

In any of these cases, the failure to confirm the experiments must be reported. Although this is acutely embarrassing for the original investigators, it represent one of the great strengths of science: its **built-in system for self-correction**.

Scientific Fraud

In the vast majority of cases, irreproducible results in science are caused by honest errors. On rare occasions, however, laboratory reports cannot be confirmed because they are fraudulent. This is distressing to all concerned. If such a fraud becomes widely known, it is also likely to cause a great deal of excitement among the general public. I believe, however, that rather than casting

a cloud over the scientific enterprise, these rare aberrations reveal its great strength.

There is probably no other area of human activity where error is detected and corrected more rapidly. I am confident that you can think of a number of other fields of human study and activity where errors have been made that went uncorrected for years and caused widespread harm. Dishonest scientists usually harm only themselves. They are disgraced; their careers often at an end. But the progress of science usually moves forward as fast as (sometimes faster than) before.

Building on the Work of Others

Only rarely does a scientific discovery spring full-blown on the scene. When it does, it is likely to create a revolution in the way scientists perceive the world around them and to open up new areas of scientific investigation. Darwin's theory of evolution and Mendel's rules of inheritance are examples of such revolutionary developments.

Most science, however, consists of adding another brick to an edifice that has been slowly and painstakingly constructed by prior work. In fact, it is possible to construct a genealogical tree that traces the historical development of any scientific discovery (even, to a degree, Darwin's and Mendel's). The way in which science builds on the work of others is another illustration of what a communal activity science is.

The development of a new **technique** often lays the foundation for rapid advances along many different scientific avenues. Just consider the advances in biology that discovery of the light microscope and, later, the electron microscope have made possible. Throughout these pages, there are many examples of experimental procedures. Each was developed to solve a particular problem. However, each was then taken up by workers in other laboratories and applied to their problems.

In a similar way, the creation of a new explanation (**hypothesis**) in a scientific field often stimulates workers in related fields to reexamine their own field in the light of the new ideas. Darwin's theory of evolution, for example, has had an enormous impact on virtually every subspecialty in biology (and in other fields as well). To this very day, biologists in specialties as different as biochemistry and animal behavior are guided in their work by evolutionary theory.

Basic Versus Applied Science

The distinction between basic and applied science is more one of goals than of methods. The same rules and standards apply to each. However, the motivation behind the work is somewhat different. Researchers in applied science have before them a practical problem to be solved. Much of the research that goes on in medicine and in agriculture is applied. The researcher in basic science, on the other hand, is primarily driven by curiosity - the desire to find out more about how nature works. Both types of research are not only honorable and demanding professions, but they are mutually dependent as well.

- Applied science repeatedly loses momentum without periodic infusions of fresh ideas and discoveries from basic research. (The light bulb would never have been discovered in the research and development (R and D) department of a candle manufacturer!)
- On the other hand, much basic research has depended on the development of new tools and instruments and, more often than not, these have been developed in laboratories devoted to applied research.

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20.4: Scientific Papers

Science is a communal activity. Only as new facts and hypotheses are taken up by the entire community of interested scientists do these facts and hypotheses become part of science. Therefore, one of the major responsibilities of scientists is to see that their work is reported to all those who might be interested.

Often this is done by word of mouth when scientists of similar interests gather together at meetings. But to be assured of a permanent place in the scientific edifice, the work is reported in a paper submitted to a scientific journal. In most cases, the paper will not be accepted for publication until it has been approved by several knowledgeable scientists from other laboratories who serve as **referees**. Often they will suggest editorial changes in the paper or even additional experiments that should be done before the paper is accepted for publication.

Papers in biology are usually divided into several sections (not necessarily in this order).

Summary or abstract

This section includes only the essence of the other sections. It should be as brief as possible, telling the reader what the goal of the experiment was, what was found, and the significance of the findings. The abstract is often placed at the beginning of the paper rather than at its end.

Introduction

This section of the paper describes the scientific question or problem that was the subject of the investigation. The introduction also includes references to earlier reports of these and other scientists that have served as the foundation for the present work.

Results

Here the authors report what happened in their experiments. This report is usually supplemented with graphs, tables, and photographs.

Discussion

Here the authors point out what they think is the significance of their findings. This is the place to show that the results are compatible with certain hypotheses and less compatible, or even incompatible, with others. If the results contradict the results of similar experiments in other laboratories, the discrepancies are noted here, and an attempt may be made to reconcile the differences.

Materials and Methods

Here are precisely described the materials used (e.g., strains of organism, source of the reagents) and all the methods followed. The goal of this section is to give all the details necessary for workers in other laboratories to be able to **repeat the experiments exactly**. When many complex procedures are involved, it is acceptable to refer to earlier papers describing these methods in greater detail.

Acknowledgments

In this brief but important section, the authors give credit to those who have assisted them in the work. These usually include technicians (who may have actually performed most of the experiments!) and other scientists who donated materials for the experiments and/or gave advice about them.

References

This section gives a careful listing of all earlier scientific work referred to in the main body of the paper. Most of the references are to other scientific papers. Each reference should provide enough information so that another person can locate the document. This means that each reference should include the name(s) of the author(s), the journal or book in which the report appears, and the year of publication. In the case of scientific journals, the volume number in which the paper appears and the page number on which the paper begins should be included. Sometimes the full title is given as well, although scientific papers often have such long titles that this is omitted from the reference.

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20.5: Statistical Methods

This page discusses how biologists manage numerical data by comparing discrete and continuous variables. It covers relevant calculations such as mean, standard deviation, and standard error of the mean (S.E.M.) to evaluate data reliability. Confidence limits are highlighted for assessing the probable range of a population's true mean, typically at a 95% confidence level.

What do the data tell us?

There are two kinds of numerical data acquired by biologists:

- counting; e.g. the number of females in a population
- measuring a **continuous variable** such as length or weight

In the first case, everyone can agree on the "true" value. In the second case, the measured values always reflect a range, the size of which is determined by such factors as

- precision of the measuring instrument and
- individual variability among the objects being measured.

How are such data handled?

Calculating the Standard Deviation

The first step is to calculate a mean (average) for all the members of the set. This is the sum of all the readings divided by the number of readings taken.

But consider the data sets:

46,42,44,45,43 and **52,80,22,30,36**

Both give the same mean (44), but I'm sure that you can see intuitively that an experimenter would have much more confidence in a mean derived from the first set of readings than one derived from the second.

One way to quantify the spread of values in a set of data is to calculate a **standard deviation** (S) using the equation

$$s = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}} \tag{20.5.1}$$

where ("x minus x-bar)^2 is the square of the difference between each individual measurement (x) and the mean ("x-bar") of the measurements. The symbol sigma indicates the sum of these, and **n** is the number of individual measurements.

Using the first data set, we calculate a standard deviation of 1.6.

X	$\bar{X}$	$(X - \bar{X})$	$(X - \bar{X})^2$
46	44	2	4
42	44	-2	4
44	44	0	0
45	44	1	1
43	44	-1	1
$\bar{X} = 44$			$\Sigma = 10$

$$S = \sqrt{\frac{\Sigma (X - \bar{X})^2}{n - 1}} = 1.6$$

The second data set produces a standard deviation of 22.9.

X	$\bar{X}$	$(X - \bar{X})$	$(X - \bar{X})^2$
52	44	8	64
80	44	36	1296
22	44	-22	484
30	44	-14	196
36	44	-8	64
$\bar{X} = 44$			$\Sigma = 2104$

$$S = \sqrt{\frac{\Sigma (X - \bar{X})^2}{n - 1}} = 22.9$$

(Many inexpensive hand-held calculators are programmed to do this job for you when you simply enter the values for X.)

Standard Error of the Mean

In our two sets of 5 measurements, both data sets give a mean of 44. But both groups are very small. How confident can we be that if we repeated the measurements thousands of times, both groups would continue to give a mean of 44? To estimate this, we calculate the standard error of the mean (S.E.M. or $S_{x\text{-bar}}$) using the equation

$$S_{\bar{x}} = \frac{S}{\sqrt{n}} \tag{20.5.2}$$

where **S** is the standard deviation and **n** is the number of measurements.

In our first data set, the S.E.M. is 0.7.

$$S_{\bar{x}} = \frac{S}{\sqrt{5}} = \frac{1.6}{2.23} = 0.7 \tag{20.5.3}$$

In the second group it is 10.3.

$$S_{\bar{x}} = \frac{S}{\sqrt{5}} = \frac{22.9}{2.23} = 10.3 \tag{20.5.4}$$

95%Confidence Limits

It turns out that there is a 68% probability that the "true" mean value of any effect being measured falls between +1 and –1 standard error (S.E.M.). Since this is not a very strong probability, most workers prefer to extend the range to limits within which they can be 95% confident that the "true" value lies. This range is roughly between –2 and +2 times the standard error.

So

- for our first group, 0.7 x 2 = 1.4
- for our second group, 10.3 x 2 = 20.6

So

- if our first group is representative of the entire population, we are 95% confident that the "true" mean lies somewhere between 42.6 and 45.4 (44 ± 1.4 or 42.6 ≤ 44 ≤ 45.4).
- for our second group, we are 95% confident that the "true" mean lies somewhere between 23.4 and 64.6 (44 ± 20.6 or 23.4 ≤ 44 ≤ 64.6).

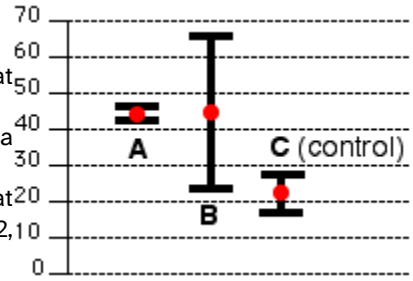
Put another way, when the mean is presented along with its 95% confidence limits, the workers are saying that there is only a 1 in 20 chance that the "true" mean value lies outside those limits.

Put still another way: the probability (**p**) that the mean value lies outside those limits is less than 1 in 20 (**p** = <0.05).

An example:

Assume that

- the first data set ("**A**") (46,42,44,45,43) represents measurements of five animals that have been given a particular treatment and
- the second data set ("**B**") (52,80,22,30,36) measurements of five other animals given a different treatment.
- A third set ("**C**") of five animals was used as controls; they were given no treatment at all, and their measurements were 20,23,24,19,24. The mean of the control group is 22, and the standard error is 2.1.



Did treatment A have a significant effect? Did treatment B?

The graph shows the mean for each data set (red dots). The dark lines represent the 95% confidence limits (± 2 standard errors).

Although both experimental means (A and B) are twice as large as the control mean, only the results in A are significant. The "true" value of B could even be simply that of the untreated animals, the controls (C).

Rejecting the null hypothesis

In principle, a scientist designs an experiment to disprove, or not, that an observed effect is due to chance alone. This is called **rejecting the null hypothesis**.

The value **p** is the probability that there is no difference between the experimental and the controls; that is, that the null hypothesis is correct. So if the probability that the experimental mean differs from the control mean is greater than 0.05, then the difference is usually not considered significant. If **p** = <0.05, the difference is considered significant, and the null hypothesis is rejected.

In our hypothetical example, the difference between the experimental group **A** and the controls (**C**) appears to be significant; that between **B** and the controls does not.

Narrowing the confidence limits

Two approaches can be taken to narrow the confidence limits.

- enlarge the size of the sample being measured (increases **n**)
- find ways to reduce the fluctuation of measurements about the mean.

The second goal is often much more difficult to achieve; if it proves impossible, perhaps the null hypothesis is right after all!

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20.6: Drugs

This page outlines the drug testing process, which starts with testing numerous chemicals on lab animals and may lead to human trials through an IND application to the FDA. The human trials consist of three phases: Phase I focuses on safety in healthy volunteers, Phase II tests efficacy and side effects in patients, and Phase III collects comprehensive safety and effectiveness data. Upon successful completion, a NDA is submitted, and the drug is branded for market release.

Testing New Drugs

Thousands of chemicals, both synthetic and extracted from "natural" sources, are being examined in the hope of finding new drugs with which to combat human and veterinary diseases. The first step is to use laboratory tests to find if these substances have a significant effect on, for example:

- cells growing in tissue culture
- laboratory animals such as rats and mice.

If the drug achieves the desired effect in laboratory animals, without killing them in the process, the drug developer applies to the U. S. Food and Drug Administration for an **IND**, an **i**nvestigational **n**ew **d**rug application. Granting of an IND allows testing in **humans** to begin. This occurs in **three phases**.

Phase I

A small group (20–100) of **healthy** volunteers is given the drug to see

- if it is safe
- how quickly it is absorbed, metabolized, and excreted from the body

Phase II

A group (up to several hundred) of volunteer **patients** with the disease are given the drug to see

- how effective it is against the signs and symptoms of the disease
- what doses are best
- what side effects may occur

A **control group** of similar size is given a dummy drug (placebo). Ideally the trials are "blinded" with neither the subjects (nor the investigator) knowing which pill a subject is receiving.

Phase III

Hundreds to thousands of **patients** with the disease are given the drug to get more reliable data on its

- effectiveness
- safety
- best dose
- rare side effects

all compared with the drug(s) that are currently used for the disease.

If all goes well, the drug manufacturer applies to the Food and Drug Administration for an **NDA**, a **n**ew **d**rug **a**pplication. If it is granted, the generic name of the drug is replaced by a brand name chosen by the manufacturer. For example, one of the first drugs used against AIDS was azidodideoxythymidine (**AZT**). When placed on the market, this name was replaced by the brand name **Retrovir®**.

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