SURVEY OF BIOCHEMISTRY AND BIOTECHNOLOGY



A. Goodman
California Polytechnic State University San Luis Obispo

LibreTexts™

Survey of Biochemistry and Biotechnology

This text is disseminated via the Open Education Resource (OER) LibreTexts Project (https://LibreTexts.org) and like the thousands of other texts available within this powerful platform, it is freely available for reading, printing, and "consuming."

The LibreTexts mission is to bring together students, faculty, and scholars in a collaborative effort to provide an accessible, and comprehensive platform that empowers our community to develop, curate, adapt, and adopt openly licensed resources and technologies; through these efforts we can reduce the financial burden born from traditional educational resource costs, ensuring education is more accessible for students and communities worldwide.

Most, but not all, pages in the library have licenses that may allow individuals to make changes, save, and print this book. Carefully consult the applicable license(s) before pursuing such effects. Instructors can adopt existing LibreTexts texts or Remix them to quickly build course-specific resources to meet the needs of their students. Unlike traditional textbooks, LibreTexts' web based origins allow powerful integration of advanced features and new technologies to support learning.



LibreTexts is the adaptable, user-friendly non-profit open education resource platform that educators trust for creating, customizing, and sharing accessible, interactive textbooks, adaptive homework, and ancillary materials. We collaborate with individuals and organizations to champion open education initiatives, support institutional publishing programs, drive curriculum development projects, and more.

The LibreTexts libraries are Powered by NICE CXone Expert and was supported by the Department of Education Open Textbook Pilot Project, the California Education Learning Lab, the UC Davis Office of the Provost, the UC Davis Library, the California State University Affordable Learning Solutions Program, and Merlot. This material is based upon work supported by the National Science Foundation under Grant No. 1246120, 1525057, and 1413739.

Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation nor the US Department of Education.

Have questions or comments? For information about adoptions or adaptions contact info@LibreTexts.org or visit our main website at https://LibreTexts.org.

This text was compiled on 03/11/2026

TABLE OF CONTENTS

Licensing

1: Chemical and Biological Foundations

- 1.1: Biological Foundations
  - 1.1.1: Properties of life
  - 1.1.2: Prokaryotic Cells and Scale
  - 1.1.3: Eukaryotic Cells
- 1.2: Atoms, Ions, Molecules
- 1.3: Intermolecular Forces
- 1.4: Carbon and Functional Groups
- 1.5: Water, Equilibrium, and Buffers

2: Structure and Function- Proteins

- 2.1: Structure and Function - Amino Acids
- 2.2: Structure and Function- Proteins I
- 2.3: Structure and Function- Proteins II

3: Catalysis

- 3.1: Basic Principles of Catalysis
- 3.2: Control of Enzymatic Activity
- 3.3: Mechanisms of Catalysis

4: Structure and Function- Nucleic Acids

- 4.1: Prelude to Information Processing
- 4.2: Structure and Function - Nucleic Acids
- 4.3: DNA Replication
- 4.4: DNA Repair
- 4.5: Genes and Genomes
- 4.6: Transcription
  - 4.6.1: RNA Processing
- 4.7: RNA Processing
- 4.8: Translation
- 4.9: Gene Expression

5: Structure and Function- Carbohydrates and Lipids

- 5.1: Structure and Function- Carbohydrates
- 5.2: Structure and Function - Lipids and Their Transport
  - 5.2.1: 5.2.1 Optional- other interesting lipids
    - 5.2.1.1: Bonus- other interesting lipids
- 5.3: Basic Concepts in Membranes
- 5.4: Transport Across Membranes
  - 5.4.1: Optional deep dive in to membrane transport

- 5.5: Other Considerations in Membranes

6: Energy and Metabolism

- 6.1: Basics of Energy
- 6.2: Metabolism - Sugars
- 6.3: Citric Acid Cycle and Related Pathways
- 6.4: Electron Transport and Oxidative Phosphorylation
- 6.5: Fats and Fatty Acids
- 6.6: Amino Acids and the Urea Cycle
- 6.7: Other Lipids
- 6.8: Energy - Photophosphorylation

7: Regulation of Metabolism and Homeostasis

- 7.1: Signaling
- 7.2: How Cells Obtain Energy
  - 7.2.1: Energy and Metabolism
  - 7.2.2: Glycolysis
  - 7.2.3: Citric Acid Cycle and Oxidative Phosphorylation
  - 7.2.4: Fermentation
  - 7.2.5: Connections to Other Metabolic Pathways
  - 7.2.E: How Cells Obtain Energy (Exercises)
- 7.3: Section - place holder

8: Biotechnology and Other Applications of Biochemistry

- 8.1: Section 1-
- 8.2: Section 2-
- 8.3: Section 3-
- 8.4: Section 4-
- 8.5: Section 5-
- 8.6: Section 6-
- 8.7: The Immune System and Disease
  - 8.7.1: Viruses
  - 8.7.2: Innate Immunity
  - 8.7.3: Adaptive Immunity
  - 8.7.4: Disruptions in the Immune System
  - 8.7.E: The Immune System and Desease (Exercises)
- 8.8: Biotechnology

9: Basic Techniques

- 9.1: Cell Lysis
- 9.2: Fractionation and Chromatography Techniques
- 9.3: Electrophoresis
- 9.4: Detection, identification and quantitation of specific nucleic acids and proteins
- 9.5: Transcriptomics
- 9.6: Isolating Genes
- 9.7: Polymerase Chain Reaction (PCR)
- 9.8: Reverse Transcription

- 9.9: Genome Editing (CRISPR)
- 9.10: Membrane Dynamics (FRAP)

10: Supplemental Modules (Biochemistry)

- 10.1: DNA
  - 10.1.1: DNA as Genetic Material
  - 10.1.2: Structure of DNA and RNA
  - 10.1.3: Bacterial Restriction/Modification system
  - 10.1.4: DNA Modifying Enzymes
  - 10.1.5: DNA Replication - Introduction to Prokaryotic replication
  - 10.1.6: DNA Supercoiling and Topoisomerases
- 10.2: Bacteria
  - 10.2.1: The Bacteria - An Introduction
  - 10.2.2: Bacterial Growth and Reproduction
  - 10.2.3: Extrachromosomal elements, plasmids, selectable markers
  - 10.2.4: Central Dogma and the Genetic Code
  - 10.2.5: Gene and Operon
  - 10.2.6: The lac Operon; CAP site; DNA footprinting
  - 10.2.7: Transcriptional Regulation- Transcription termination, the trp operon
  - 10.2.8: mRNA Translation
- 10.3: 3. Biotechnology 1
  - 10.3.1: Gel Electrophoresis
  - 10.3.2: DNA Sequence Analysis
  - 10.3.3: Polymerase Chain Reaction (PCR) and Cloning of PCR Products
  - 10.3.4: Prokaryotic Expression Vectors
  - 10.3.5: Protein Sequencing, Peptide Mapping, Synthetic Genes
  - 10.3.6: cDNA and Genomic Libraries
  - 10.3.7: Protein Purification
- 10.4: 5. Lab Notes Part 1
  - 10.4.1: Basic practices and techniques, Laboratory notebooks, Presentation of data, Sections of a scientific report
  - 10.4.2: Spectrophotometry
  - 10.4.3: Quantification of Protein Concentration
  - 10.4.4: Chromatography
  - 10.4.5: Gel Electrophoresis of Proteins
  - 10.4.6: Introduction to Protein Purification
- 10.5: 6. Lab Notes Part 2
  - 10.5.1: Genetic Transformation (using bacteria and the pGLO plasmid)
  - 10.5.2: Enzyme kinetics
  - 10.5.3: Ligand binding
  - 10.5.4: Restriction Mapping
  - 10.5.5: Polymerase Chain Reaction (PCR)
  - 10.5.6: Use of PC and internet for biochemical research
- 10.6: 4. Biotechnology 2
  - 10.6.1: M13 Phage
  - 10.6.2: M13 Phage Display Libraries
  - 10.6.3: SELEX (Selective Evolution of Ligands by Exponential Enrichment)
  - 10.6.4: Protein-Protein Recognition Probed Using a Yeast Transcriptional Activator System

- 10.6.5: Molecular Imprinting

Index

Glossary

Detailed Licensing

Licensing

*A detailed breakdown of this resource's licensing can be found in **Back Matter/Detailed Licensing**.*

CHAPTER OVERVIEW

1: Chemical and Biological Foundations

1.1: Biological Foundations

1.1.1: Properties of life

1.1.2: Prokaryotic Cells and Scale

1.1.3: Eukaryotic Cells

1.2: Atoms, Ions, Molecules

1.3: Intermolecular Forces

1.4: Carbon and Functional Groups

1.5: Water, Equilibrium, and Buffers

Thumbnail: Attribution

1: Chemical and Biological Foundations is shared under a not declared license and was authored, remixed, and/or curated by LibreTexts.

SECTION OVERVIEW

1.1: Biological Foundations

1.1.1: Properties of life

1.1.2: Prokaryotic Cells and Scale

1.1.3: Eukaryotic Cells

Template:HideTOC

Contributors and Attributions

- Prof. Henry Jakubowski (College of St. Benedict/St. John's University)

1.1: Biological Foundations is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by LibreTexts.

1.1.1: Properties of life

Skills to Develop

- Identify and describe the properties of life
- Describe the levels of organization among living things
- Recognize and interpret a phylogenetic tree
- List examples of different sub disciplines in biology

Biology is the science that studies life, but what exactly is life? This may sound like a silly question with an obvious response, but it is not always easy to define life. For example, a branch of biology called virology studies viruses, which exhibit some of the characteristics of living entities but lack others. It turns out that although viruses can attack living organisms, cause diseases, and even reproduce, they do not meet the criteria that biologists use to define life. Consequently, virologists are not biologists, strictly speaking. Similarly, some biologists study the early molecular evolution that gave rise to life; since the events that preceded life are not biological events, these scientists are also excluded from biology in the strict sense of the term.

From its earliest beginnings, biology has wrestled with three questions: What are the shared properties that make something "alive"? And once we know something is alive, how do we find meaningful levels of organization in its structure? And, finally, when faced with the remarkable diversity of life, how do we organize the different kinds of organisms so that we can better understand them? As new organisms are discovered every day, biologists continue to seek answers to these and other questions.

Properties of Life

All living organisms share several key characteristics or functions: order, sensitivity or response to the environment, reproduction, adaptation, growth and development, regulation, homeostasis, energy processing, and evolution. When viewed together, these nine characteristics serve to define life.

Order

Organisms are highly organized, coordinated structures that consist of one or more cells. Even very simple, single-celled organisms are remarkably complex: inside each cell, atoms make up molecules; these in turn make up cell organelles and other cellular inclusions. In multicellular organisms (Figure **1.1.1.1**), similar cells form tissues. Tissues, in turn, collaborate to create organs (body structures with a distinct function). Organs work together to form organ systems.



Figure **1.1.1.1**: A toad represents a highly organized structure consisting of cells, tissues, organs, and organ systems. (credit: "Ivengo"/Wikimedia Commons)

Sensitivity or Response to Stimuli

Organisms respond to diverse stimuli. For example, plants can bend toward a source of light, climb on fences and walls, or respond to touch (Figure **1.1.1.2**). Even tiny bacteria can move toward or away from chemicals (a process called *chemotaxis*) or light (*phototaxis*). Movement toward a stimulus is considered a positive response, while movement away from a stimulus is considered a negative response.



Figure **1.1.1.2**: The leaves of this sensitive plant (*Mimosa pudica*) will instantly droop and fold when touched. After a few minutes, the plant returns to normal. (credit: Alex Lomas)

Link to Learning

Video: Watch this video to see how plants respond to a stimulus—from opening to light, to wrapping a tendril around a branch, to capturing prey.

Reproduction

Single-celled organisms reproduce by first duplicating their DNA, and then dividing it equally as the cell prepares to divide to form two new cells. Multicellular organisms often produce specialized reproductive germline cells that will form new individuals. When reproduction occurs, genes containing DNA are passed along to an organism's offspring. These genes ensure that the offspring will belong to the same species and will have similar characteristics, such as size and shape.

Growth and Development

Organisms grow and develop following specific instructions coded for by their genes. These genes provide instructions that will direct cellular growth and development, ensuring that a species' young (Figure **1.1.1.3**) will grow up to exhibit many of the same characteristics as its parents.



Figure 1.1.1.3: Although no two look alike, these kittens have inherited genes from both parents and share many of the same characteristics. (credit: Rocky Mountain Feline Rescue)

Regulation

Even the smallest organisms are complex and require multiple regulatory mechanisms to coordinate internal functions, respond to stimuli, and cope with environmental stresses. Two examples of internal functions regulated in an organism are nutrient transport and blood flow. Organs (groups of tissues working together) perform specific functions, such as carrying oxygen throughout the body, removing wastes, delivering nutrients to every cell, and cooling the body.

Homeostasis

In order to function properly, cells need to have appropriate conditions such as proper temperature, pH, and appropriate concentration of diverse chemicals. These conditions may, however, change from one moment to the next. Organisms are able to maintain internal conditions within a narrow range almost constantly, despite environmental changes, through homeostasis (literally, “steady state”)—the ability of an organism to maintain constant internal conditions. For example, an organism needs to regulate body temperature through a process known as thermoregulation. Organisms that live in cold climates, such as the polar bear (Figure 1.1.1.4), have body structures that help them withstand low temperatures and conserve body heat. Structures that aid in this type of insulation include fur, feathers, blubber, and fat. In hot climates, organisms have methods (such as perspiration in humans or panting in dogs) that help them to shed excess body heat.



Figure 1.1.1.4: Polar bears (*Ursus maritimus*) and other mammals living in ice-covered regions maintain their body temperature by generating heat and reducing heat loss through thick fur and a dense layer of fat under their skin. (credit: “longhorndave”/Flickr)

Energy Processing

All organisms use a source of energy for their metabolic activities. Some organisms capture energy from the sun and convert it into chemical energy in food; others use chemical energy in molecules they take in as food (Figure 1.1.1.5).



Figure 1.1.1.5: The California condor (*Gymnogyps californianus*) uses chemical energy derived from food to power flight. California condors are an endangered species; this bird has a wing tag that helps biologists identify the individual. (credit: Pacific Southwest Region U.S. Fish and Wildlife Service)

Levels of Organization of Living Things

Living things are highly organized and structured, following a hierarchy that can be examined on a scale from small to large. The atom is the smallest and most fundamental unit of matter. It consists of a nucleus surrounded by electrons. Atoms form molecules. A molecule is a chemical structure consisting of at least two atoms held together by one or more chemical bonds. Many molecules that are biologically important are macromolecules, large molecules that are typically formed by polymerization (a polymer is a large molecule that is made by combining smaller units called monomers, which are simpler than macromolecules). An example of a macromolecule is deoxyribonucleic acid (DNA) (Figure 1.1.1.6), which contains the instructions for the structure and functioning of all living organisms.

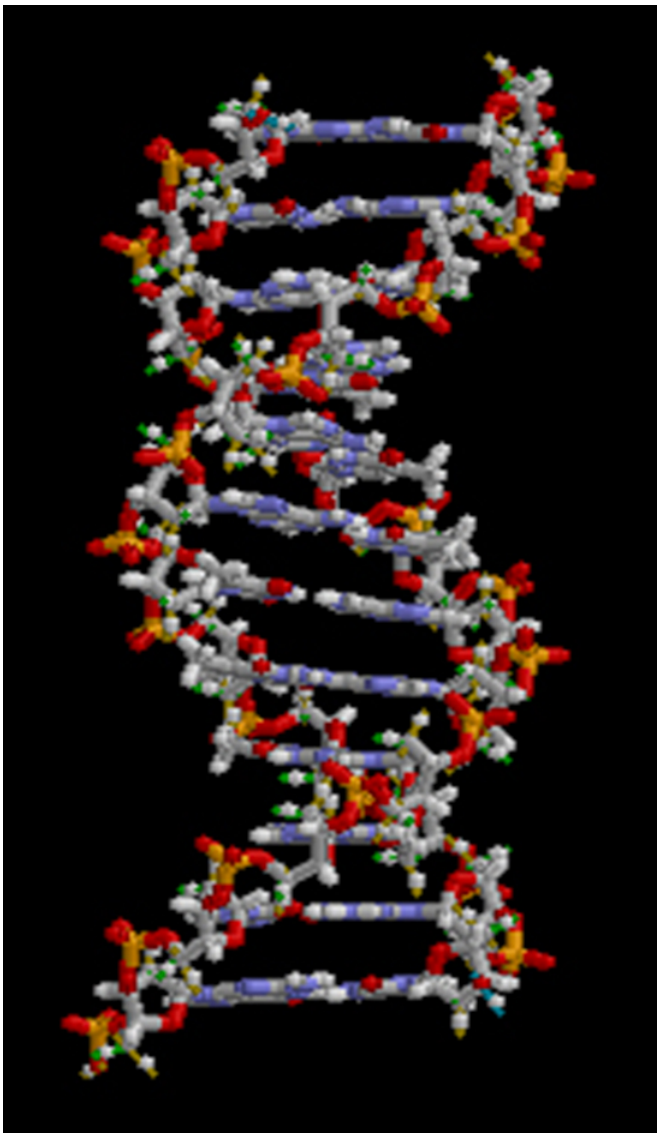


Figure 1.1.1.6: All molecules, including this DNA molecule, are composed of atoms. (credit: “brian0918”/Wikimedia Commons)

Link to Learning

Video: Watch this video that animates the three-dimensional structure of the DNA molecule shown in Figure 1.1.1.6.

Some cells contain aggregates of macromolecules surrounded by membranes; these are called organelles. Organelles are small structures that exist within cells. Examples of organelles include mitochondria and chloroplasts, which carry out indispensable functions: mitochondria produce energy to power the cell, while chloroplasts enable green plants to utilize the energy in sunlight to make sugars. All living things are made of cells; the cell itself is the smallest fundamental unit of structure and function in living organisms. (This requirement is why viruses are not considered living: they are not made of cells. To make new viruses, they have to invade and hijack the reproductive mechanism of a living cell; only then can they obtain the materials they need to reproduce.)

Some organisms consist of a single cell and others are multicellular. Cells are classified as prokaryotic or eukaryotic. Prokaryotes are single-celled or colonial organisms that do not have membrane-bound nuclei; in contrast, the cells of eukaryotes do have membrane-bound organelles and a membrane-bound nucleus.

In larger organisms, cells combine to make tissues, which are groups of similar cells carrying out similar or related functions. Organs are collections of tissues grouped together performing a common function. Organs are present not only in animals but also in plants. An organ system is a higher level of organization that consists of functionally related organs. Mammals have many organ systems. For instance, the circulatory system transports blood through the body and to and from the lungs; it includes organs such as the heart and blood vessels. Organisms are individual living entities. For example, each tree in a forest is an organism. Single-celled prokaryotes and single-celled eukaryotes are also considered organisms and are typically referred to as microorganisms.

All the individuals of a species living within a specific area are collectively called a population. For example, a forest may include many pine trees. All of these pine trees represent the population of pine trees in this forest. Different populations may live in the same specific area. For example, the forest with the pine trees includes populations of flowering plants and also insects and microbial populations. A community is the sum of populations inhabiting a particular area. For instance, all of the trees, flowers, insects, and other populations in a forest form the forest's community. The forest itself is an ecosystem. An ecosystem consists of all the living things in a particular area together with the abiotic, non-living parts of that environment such as nitrogen in the soil or rain water. At the highest level of organization (Figure 1.1.1.7), the biosphere is the collection of all ecosystems, and it represents the zones of life on earth. It includes land, water, and even the atmosphere to a certain extent.

Art Connection

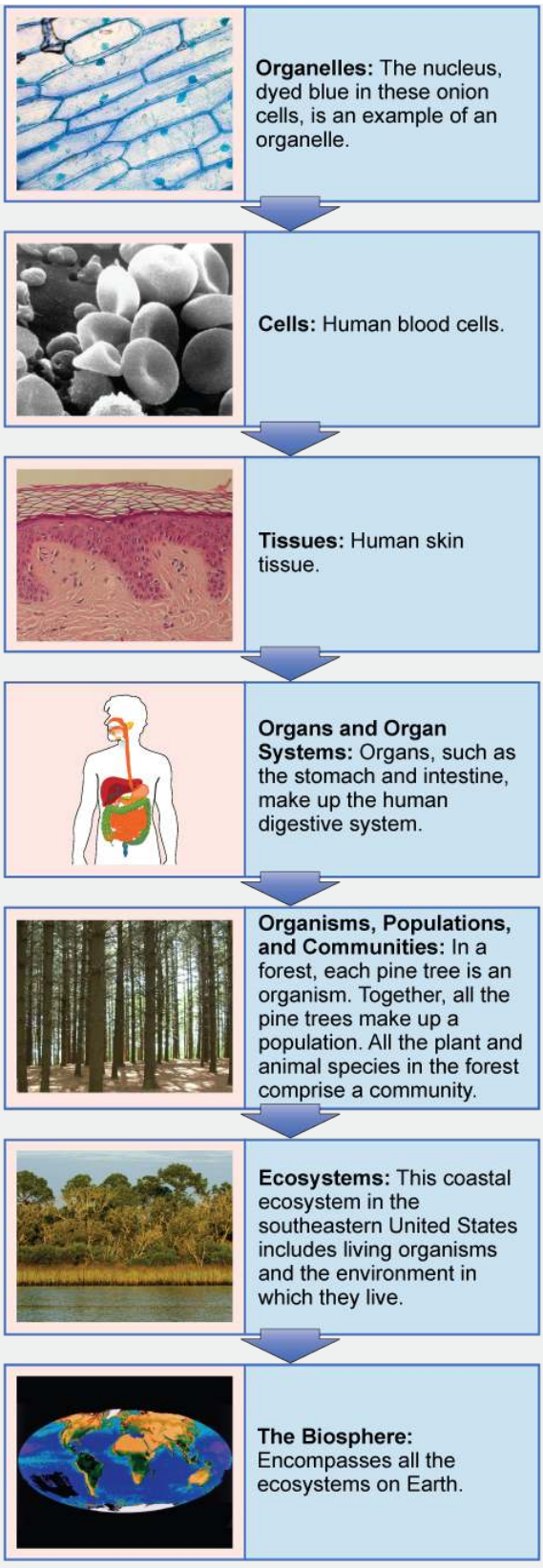


Figure **1.1.1.7**: The biological levels of organization of living things are shown. From a single organelle to the entire biosphere, living organisms are parts of a highly structured hierarchy. (credit “organelles”: modification of work by Umberto Salvagnin; credit “cells”: modification of work by Bruce Wetzel, Harry Schaefer/ National Cancer Institute; credit “tissues”: modification of work by Kilbad; Fama Clamosa; Mikael Häggström; credit “organs”: modification of work by Mariana Ruiz Villareal; credit “organisms”: modification of work by "Crystal"/Flickr;

credit “ecosystems”: modification of work by US Fish and Wildlife Service Headquarters; credit “biosphere”: modification of work by NASA)

Which of the following statements is false?

A. Tissues exist within organs which exist within organ systems.
B. Communities exist within populations which exist within ecosystems.
C. Organelles exist within cells which exist within tissues.
D. Communities exist within ecosystems which exist in the biosphere.

The Diversity of Life

The fact that biology, as a science, has such a broad scope has to do with the tremendous diversity of life on earth. The source of this diversity is evolution, the process of gradual change during which new species arise from older species. Evolutionary biologists study the evolution of living things in everything from the microscopic world to ecosystems.

The evolution of various life forms on Earth can be summarized in a phylogenetic tree (Figure 1.1.1.8). A phylogenetic tree is a diagram showing the evolutionary relationships among biological species based on similarities and differences in genetic or physical traits or both. A phylogenetic tree is composed of nodes and branches. The internal nodes represent ancestors and are points in evolution when, based on scientific evidence, an ancestor is thought to have diverged to form two new species. The length of each branch is proportional to the time elapsed since the split.

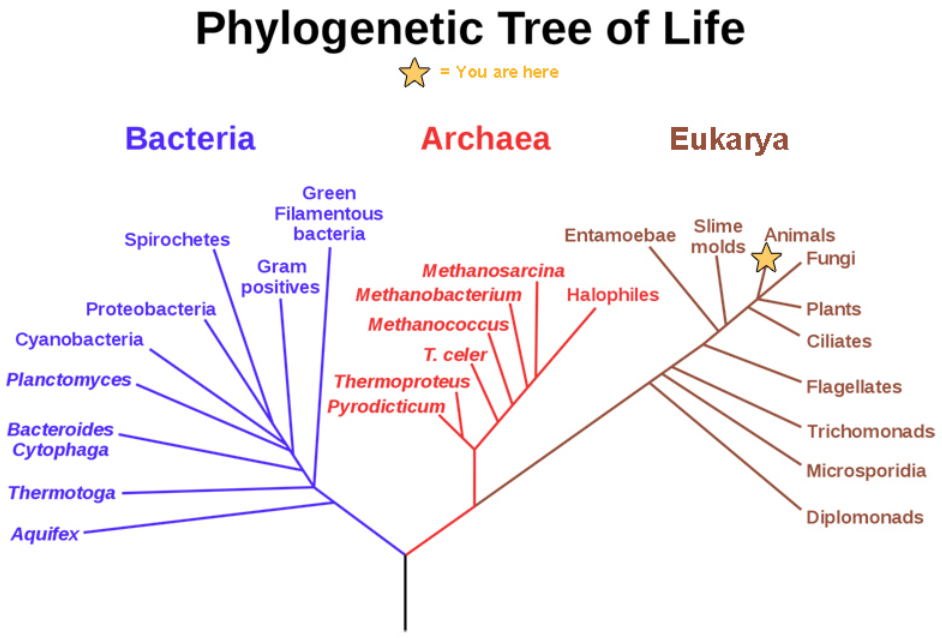


Figure 1.1.1.8: This phylogenetic tree was constructed by microbiologist Carl Woese using data obtained from sequencing ribosomal RNA genes. The tree shows the separation of living organisms into three domains: Bacteria, Archaea, and Eukarya. Bacteria and Archaea are prokaryotes, single-celled organisms lacking intracellular organelles. (credit: Eric Gaba; NASA Astrobiology Institute)

Evolution Connection: Carl Woese and the Phylogenetic Tree

In the past, biologists grouped living organisms into five kingdoms: animals, plants, fungi, protists, and bacteria. The organizational scheme was based mainly on physical features, as opposed to physiology, biochemistry, or molecular biology, all of which are used by modern systematics. The pioneering work of American microbiologist Carl Woese in the early 1970s has shown, however, that life on Earth has evolved along three lineages, now called domains—Bacteria, Archaea, and Eukarya. The first two are prokaryotic cells with microbes that lack membrane-enclosed nuclei and organelles. The third domain contains the eukaryotes and includes unicellular microorganisms together with the four original kingdoms (excluding bacteria). Woese defined Archaea as a new domain, and this resulted in a new taxonomic tree (Figure 1.1.1.8). Many organisms belonging

to the Archaea domain live under extreme conditions and are called extremophiles. To construct his tree, Woese used genetic relationships rather than similarities based on morphology (shape).

Woese's tree was constructed from comparative sequencing of the genes that are universally distributed, present in every organism, and conserved (meaning that these genes have remained essentially unchanged throughout evolution). Woese's approach was revolutionary because comparisons of physical features are insufficient to differentiate between the prokaryotes that appear fairly similar in spite of their tremendous biochemical diversity and genetic variability (Figure **1.1.1.9**). The comparison of homologous DNA and RNA sequences provided Woese with a sensitive device that revealed the extensive variability of prokaryotes, and which justified the separation of the prokaryotes into two domains: bacteria and archaea.

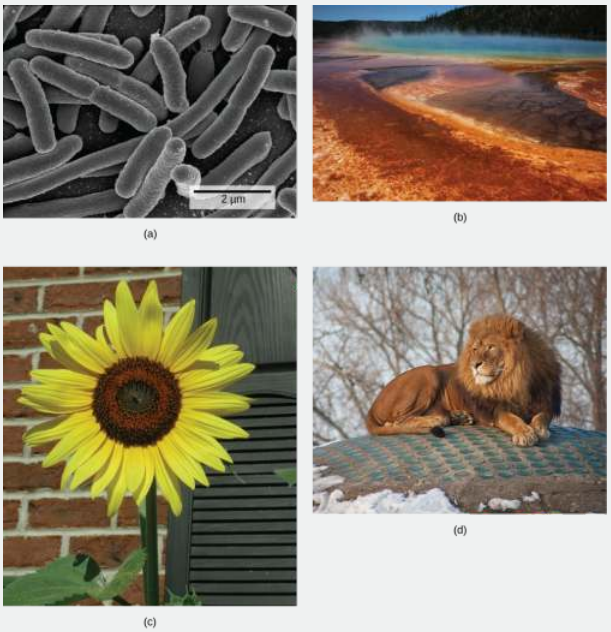


Figure **1.1.1.9**: These images represent different domains. The (a) bacteria in this micrograph belong to Domain Bacteria, while the (b) extremophiles (not visible) living in this hot vent belong to Domain Archaea. Both the (c) sunflower and (d) lion are part of Domain Eukarya. (credit a: modification of work by Drew March; credit b: modification of work by Steve Jurvetson; credit c: modification of work by Michael Arrighi; credit d: modification of work by Leszek Leszcynski)

Branches of Biological Study

The scope of biology is broad and therefore contains many branches and subdisciplines. Biologists may pursue one of those subdisciplines and work in a more focused field. For instance, molecular biology and biochemistry study biological processes at the molecular and chemical level, including interactions among molecules such as DNA, RNA, and proteins, as well as the way they are regulated. Microbiology, the study of microorganisms, is the study of the structure and function of single-celled organisms. It is quite a broad branch itself, and depending on the subject of study, there are also microbial physiologists, ecologists, and geneticists, among others.

Career Connection: Forensic Scientist

Forensic science is the application of science to answer questions related to the law. Biologists as well as chemists and biochemists can be forensic scientists. Forensic scientists provide scientific evidence for use in courts, and their job involves examining trace materials associated with crimes. Interest in forensic science has increased in the last few years, possibly because of popular television shows that feature forensic scientists on the job. Also, the development of molecular techniques and the establishment of DNA databases have expanded the types of work that forensic scientists can do. Their job activities are primarily related to crimes against people such as murder, rape, and assault. Their work involves analyzing samples such as hair, blood, and other body fluids and also processing DNA (Figure **1.1.1.10**) found in many different environments and materials. Forensic scientists also analyze other biological evidence left at crime scenes, such as insect larvae or pollen grains. Students who want to pursue careers in forensic science will most likely be required to take chemistry and biology courses as well as some intensive math courses.



Figure 1.1.1.10: This forensic scientist works in a DNA extraction room at the U.S. Army Criminal Investigation Laboratory at Fort Gillem, GA. (credit: United States Army CID Command Public Affairs)

Another field of biological study, neurobiology, studies the biology of the nervous system, and although it is considered a branch of biology, it is also recognized as an interdisciplinary field of study known as neuroscience. Because of its interdisciplinary nature, this subdiscipline studies different functions of the nervous system using molecular, cellular, developmental, medical, and computational approaches.



Figure 1.1.1.11:Researchers work on excavating dinosaur fossils at a site in Castellón, Spain. (credit: Mario Modesto)

Paleontology, another branch of biology, uses fossils to study life's history (Figure 1.1.1.11). Zoology and botany are the study of animals and plants, respectively. Biologists can also specialize as biotechnologists, ecologists, or physiologists, to name just a few areas. This is just a small sample of the many fields that biologists can pursue.

Biology is the culmination of the achievements of the natural sciences from their inception to today. Excitingly, it is the cradle of emerging sciences, such as the biology of brain activity, genetic engineering of custom organisms, and the biology of evolution that uses the laboratory tools of molecular biology to retrace the earliest stages of life on earth. A scan of news headlines—whether reporting on immunizations, a newly discovered species, sports doping, or a genetically-modified food—demonstrates the way biology is active in and important to our everyday world.

Summary

Biology is the science of life. All living organisms share several key properties such as order, sensitivity or response to stimuli, reproduction, growth and development, regulation, homeostasis, and energy processing. Living things are highly organized parts of a hierarchy that includes atoms, molecules, organelles, cells, tissues, organs, and organ systems. Organisms, in turn, are grouped as populations, communities, ecosystems, and the biosphere. The great diversity of life today evolved from less-diverse ancestral

organisms over billions of years. A diagram called a phylogenetic tree can be used to show evolutionary relationships among organisms.

Biology is very broad and includes many branches and subdisciplines. Examples include molecular biology, microbiology, neurobiology, zoology, and botany, among others.

Art Connections

Figure 1.1.1.7: Which of the following statements is false?

A. Tissues exist within organs which exist within organ systems.
B. Communities exist within populations which exist within ecosystems.
C. Organelles exist within cells which exist within tissues.
D. Communities exist within ecosystems which exist in the biosphere.

Answer

Communities exist within populations which exist within ecosystems.

Glossary

atom
smallest and most fundamental unit of matter

biochemistry
study of the chemistry of biological organisms

biosphere
collection of all the ecosystems on Earth

botany
study of plants

cell
smallest fundamental unit of structure and function in living things

community
set of populations inhabiting a particular area

ecosystem
all the living things in a particular area together with the abiotic, nonliving parts of that environment

eukaryote
organism with cells that have nuclei and membrane-bound organelles

evolution
process of gradual change during which new species arise from older species and some species become extinct

homeostasis
ability of an organism to maintain constant internal conditions

macromolecule
large molecule, typically formed by the joining of smaller molecules

microbiology

study of the structure and function of microorganisms

molecule
chemical structure consisting of at least two atoms held together by one or more chemical bonds

molecular biology
study of biological processes and their regulation at the molecular level, including interactions among molecules such as DNA, RNA, and proteins

neurobiology
study of the biology of the nervous system

organ
collection of related tissues grouped together performing a common function

organ system
level of organization that consists of functionally related interacting organs

organelle
small structures that exist within cells and carry out cellular functions

organism
individual living entity

paleontology
study of life's history by means of fossils

phylogenetic tree
diagram showing the evolutionary relationships among various biological species based on similarities and differences in genetic or physical traits or both; in essence, a hypothesis concerning evolutionary connections

population
all of the individuals of a species living within a specific area

prokaryote
single-celled organism that lacks organelles and does not have nuclei surrounded by a nuclear membrane

tissue
group of similar cells carrying out related functions

zoology
study of animals

1.1.1: Properties of life is shared under a CC BY license and was authored, remixed, and/or curated by LibreTexts.

- **1.2: Themes and Concepts of Biology** is licensed CC BY 4.0.

1.1.2: Prokaryotic Cells and Scale

Cells fall into one of two broad categories: prokaryotic and eukaryotic. The predominantly single-celled organisms of the domains Bacteria and Archaea are classified as prokaryotes (*pro-* = before; *-karyon-* = nucleus). Animal cells, plant cells, fungi, and protists are eukaryotes (*eu-* = true).

Components of Prokaryotic Cells

All cells share four common components: 1) a plasma membrane, an outer covering that separates the cell's interior from its surrounding environment; 2) cytoplasm, consisting of a jelly-like region within the cell in which other cellular components are found; 3) DNA, the genetic material of the cell; and 4) ribosomes, particles that synthesize proteins. However, prokaryotes differ from eukaryotic cells in several ways.

A prokaryotic cell is a simple, single-celled (unicellular) organism that lacks a nucleus, or any other membrane-bound organelle. We will shortly come to see that this is significantly different in eukaryotes. Prokaryotic DNA is found in the central part of the cell: a darkened region called the nucleoid (Figure **1.1.2.1**).

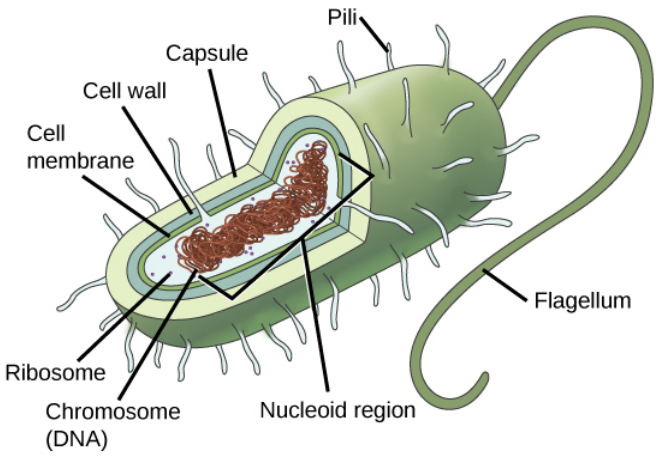


Figure 1.1.2.1: This figure shows the generalized structure of a prokaryotic cell.

Unlike Archaea and eukaryotes, bacteria have a cell wall made of peptidoglycan, comprised of sugars and amino acids, and many have a polysaccharide capsule (Figure **1.1.2.1**). The cell wall acts as an extra layer of protection, helps the cell maintain its shape, and prevents dehydration. The capsule enables the cell to attach to surfaces in its environment. Some prokaryotes have flagella, pili, or fimbriae. Flagella are used for locomotion, while most pili are used to exchange genetic material during a type of reproduction called conjugation.

Eukaryotic Cells

In nature, the relationship between form and function is apparent at all levels, including the level of the cell, and this will become clear as we explore eukaryotic cells. The principle "form follows function" is found in many contexts. For example, birds and fish have streamlined bodies that allow them to move quickly through the medium in which they live, be it air or water. It means that, in general, one can deduce the function of a structure by looking at its form, because the two are matched.

A eukaryotic cell is a cell that has a membrane-bound nucleus and other membrane-bound compartments or sacs, called organelles, which have specialized functions. The word eukaryotic means "true kernel" or "true nucleus," alluding to the presence of the membrane-bound nucleus in these cells. The word "organelle" means "little organ," and, as already mentioned, organelles have specialized cellular functions, just as the organs of your body have specialized functions.

Cell Size

At 0.1–5.0 µm in diameter, prokaryotic cells are significantly smaller than eukaryotic cells, which have diameters ranging from 10–100 µm (Figure **1.1.2.2**). The small size of prokaryotes allows ions and organic molecules that enter them to quickly spread to other parts of the cell. Similarly, any wastes produced within a prokaryotic cell can quickly move out. However, larger eukaryotic cells

have evolved different structural adaptations to enhance cellular transport. Indeed, the large size of these cells would not be possible without these adaptations. In general, cell size is limited because volume increases much more quickly than does cell surface area. As a cell becomes larger, it becomes more and more difficult for the cell to acquire sufficient materials to support the processes inside the cell, because the relative size of the surface area across which materials must be transported declines.

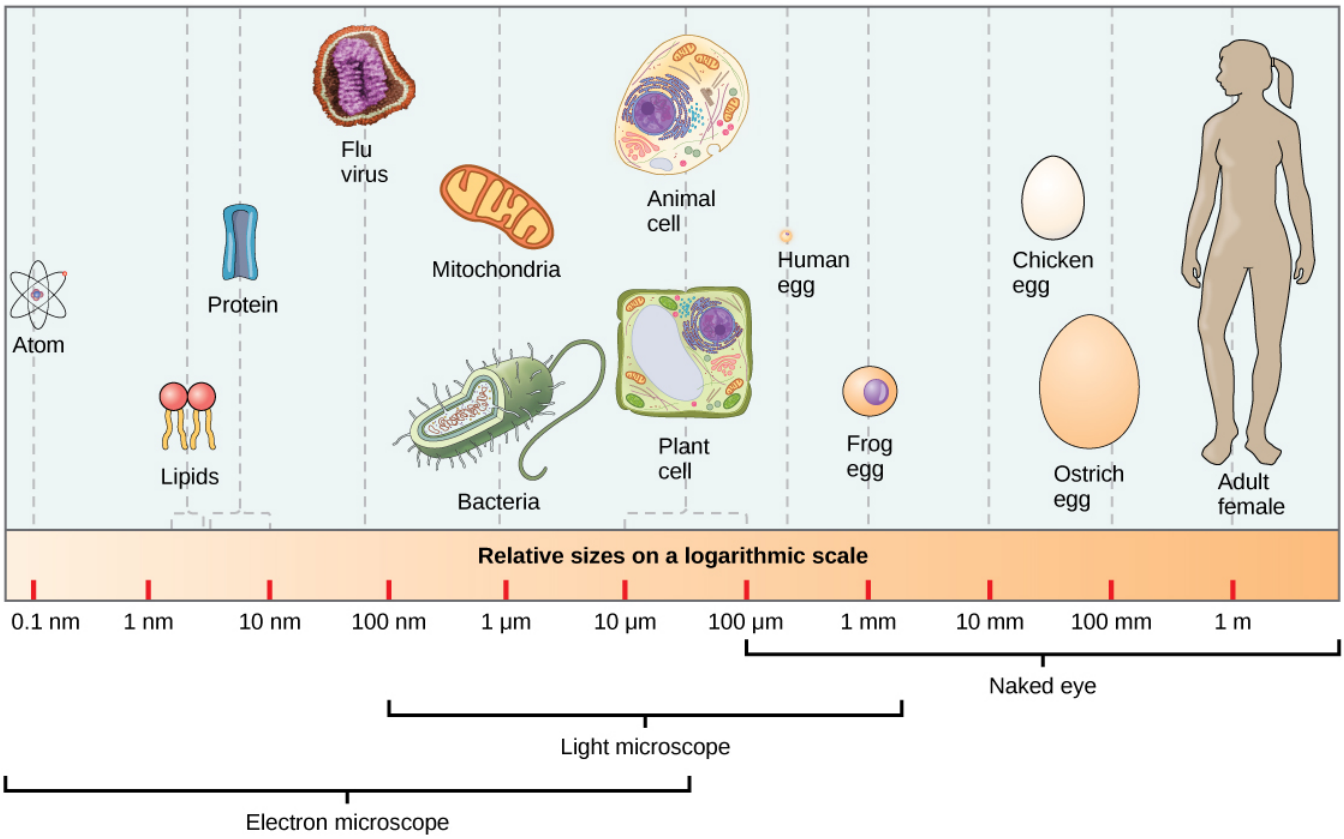


Figure 1.1.2.2: This figure shows the relative sizes of different kinds of cells and cellular components. An adult human is shown for comparison.

Section Summary

Prokaryotes are predominantly single-celled organisms of the domains Bacteria and Archaea. All prokaryotes have plasma membranes, cytoplasm, ribosomes, a cell wall, DNA, and lack membrane-bound organelles. Many also have polysaccharide capsules. Prokaryotic cells range in diameter from 0.1–5.0 µm.

Like a prokaryotic cell, a eukaryotic cell has a plasma membrane, cytoplasm, and ribosomes, but a eukaryotic cell is typically larger than a prokaryotic cell, has a true nucleus (meaning its DNA is surrounded by a membrane), and has other membrane-bound organelles that allow for compartmentalization of functions. Eukaryotic cells tend to be 10 to 100 times the size of prokaryotic cells.

Glossary

eukaryotic cell

a cell that has a membrane-bound nucleus and several other membrane-bound compartments or sacs

organelle

a membrane-bound compartment or sac within a cell

prokaryotic cell

a unicellular organism that lacks a nucleus or any other membrane-bound organelle

Contributors and Attributions

- Samantha Fowler (Clayton State University), Rebecca Roush (Sandhills Community College), James Wise (Hampton University). Original content by OpenStax (CC BY 4.0; Access for free at https://cnx.org/contents/b3c1e1d2-83...4-e119a8aafbdd).

This page titled 1.1.2: Prokaryotic Cells and Scale is shared under a CC BY license and was authored, remixed, and/or curated by .

- **3.2: Comparing Prokaryotic and Eukaryotic Cells** by OpenStax is licensed CC BY 4.0.

1.1.3: Eukaryotic Cells

At this point, it should be clear that eukaryotic cells have a more complex structure than do prokaryotic cells. Organelles allow for various functions to occur in the cell at the same time. Before discussing the functions of organelles within a eukaryotic cell, let us first examine two important components of the cell: the plasma membrane and the cytoplasm.

ART CONNECTION

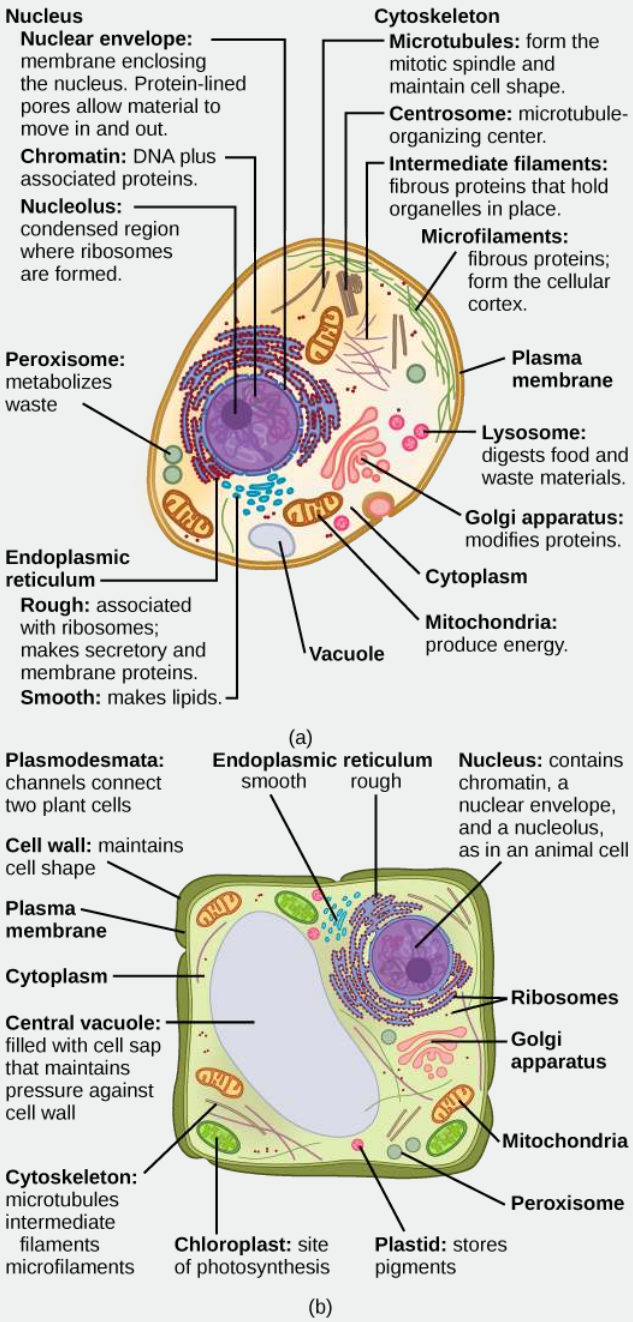


Figure 1.1.3.1: This figure shows (a) a typical animal cell and (b) a typical plant cell.

What structures does a plant cell have that an animal cell does not have? What structures does an animal cell have that a plant cell does not have?

The Plasma Membrane

Like prokaryotes, eukaryotic cells have a plasma membrane (Figure **1.1.3.2**) made up of a phospholipid bilayer with embedded proteins that separates the internal contents of the cell from its surrounding environment. A phospholipid is a lipid molecule composed of two fatty acid chains, a glycerol backbone, and a phosphate group. The plasma membrane regulates the passage of some substances, such as organic molecules, ions, and water, preventing the passage of some to maintain internal conditions, while actively bringing in or removing others. Other compounds move passively across the membrane.

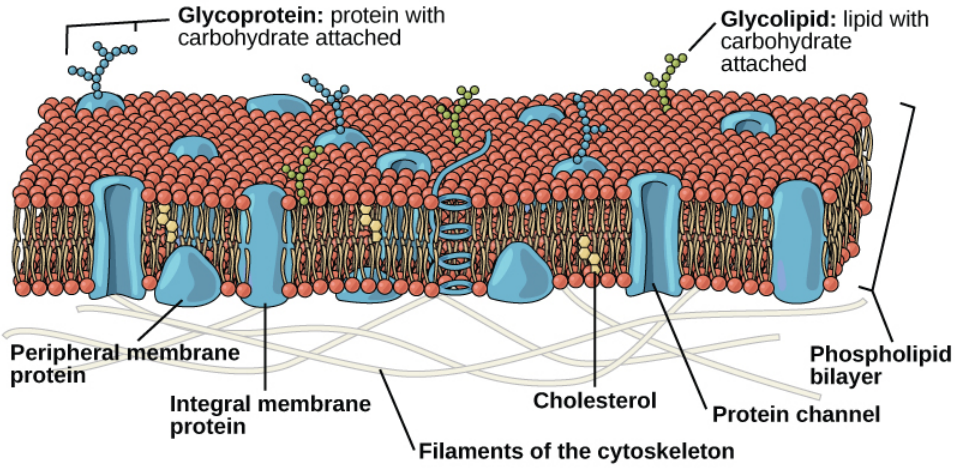


Figure 1.1.3.2: The plasma membrane is a phospholipid bilayer with embedded proteins. There are other components, such as cholesterol and carbohydrates, which can be found in the membrane in addition to phospholipids and protein.

The plasma membranes of cells that specialize in absorption are folded into fingerlike projections called microvilli (singular = microvillus). This folding increases the surface area of the plasma membrane. Such cells are typically found lining the small intestine, the organ that absorbs nutrients from digested food. This is an excellent example of form matching the function of a structure.

People with celiac disease have an immune response to gluten, which is a protein found in wheat, barley, and rye. The immune response damages microvilli, and thus, afflicted individuals cannot absorb nutrients. This leads to malnutrition, cramping, and diarrhea. Patients suffering from celiac disease must follow a gluten-free diet.

The Cytoplasm

The cytoplasm comprises the contents of a cell between the plasma membrane and the nuclear envelope (a structure to be discussed shortly). It is made up of organelles suspended in the gel-like cytosol, the cytoskeleton, and various chemicals (Figure **1.1.3.1**). Even though the cytoplasm consists of 70 to 80 percent water, it has a semi-solid consistency, which comes from the proteins within it. However, proteins are not the only organic molecules found in the cytoplasm. Glucose and other simple sugars, polysaccharides, amino acids, nucleic acids, fatty acids, and derivatives of glycerol are found there too. Ions of sodium, potassium, calcium, and many other elements are also dissolved in the cytoplasm. Many metabolic reactions, including protein synthesis, take place in the cytoplasm.

The Cytoskeleton

If you were to remove all the organelles from a cell, would the plasma membrane and the cytoplasm be the only components left? No. Within the cytoplasm, there would still be ions and organic molecules, plus a network of protein fibers that helps to maintain the shape of the cell, secures certain organelles in specific positions, allows cytoplasm and vesicles to move within the cell, and enables unicellular organisms to move independently. Collectively, this network of protein fibers is known as the cytoskeleton. There are three types of fibers within the cytoskeleton: microfilaments, also known as actin filaments, intermediate filaments, and microtubules (Figure **1.1.3.3**).

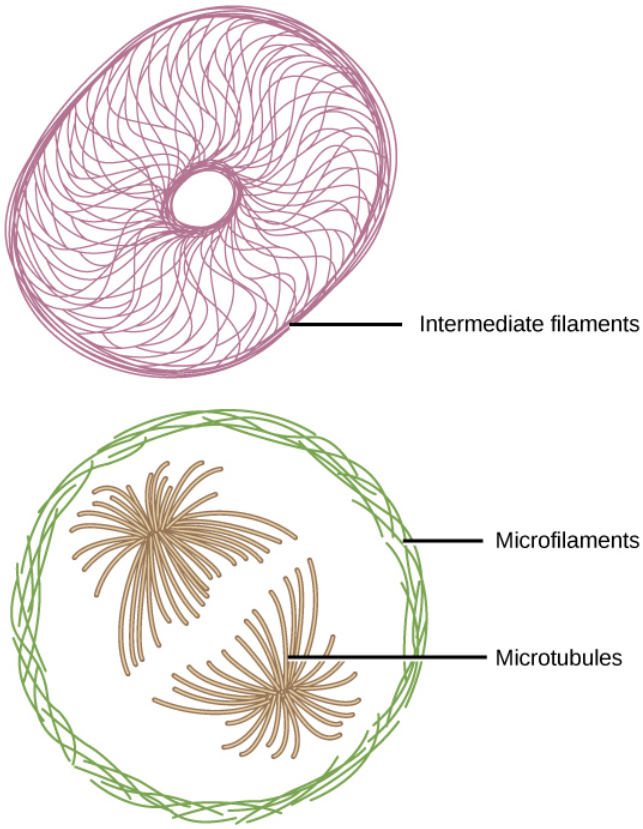


Figure 1.1.3.3: Microfilaments, intermediate filaments, and microtubules compose a cell's cytoskeleton.

Microfilaments are the thinnest of the cytoskeletal fibers and function in moving cellular components, for example, during cell division. They also maintain the structure of microvilli, the extensive folding of the plasma membrane found in cells dedicated to absorption. These components are also common in muscle cells and are responsible for muscle cell contraction. Intermediate filaments are of intermediate diameter and have structural functions, such as maintaining the shape of the cell and anchoring organelles. Keratin, the compound that strengthens hair and nails, forms one type of intermediate filament. Microtubules are the thickest of the cytoskeletal fibers. These are hollow tubes that can dissolve and reform quickly. Microtubules guide organelle movement and are the structures that pull chromosomes to their poles during cell division. They are also the structural components of flagella and cilia. In cilia and flagella, the microtubules are organized as a circle of nine double microtubules on the outside and two microtubules in the center.

The centrosome is a region near the nucleus of animal cells that functions as a microtubule-organizing center. It contains a pair of centrioles, two structures that lie perpendicular to each other. Each centriole is a cylinder of nine triplets of microtubules.

The centrosome replicates itself before a cell divides, and the centrioles play a role in pulling the duplicated chromosomes to opposite ends of the dividing cell. However, the exact function of the centrioles in cell division is not clear, since cells that have the centrioles removed can still divide, and plant cells, which lack centrioles, are capable of cell division.

Flagella and Cilia

Flagella (singular = flagellum) are long, hair-like structures that extend from the plasma membrane and are used to move an entire cell, (for example, sperm, *Euglena*). When present, the cell has just one flagellum or a few flagella. When cilia (singular = cilium) are present, however, they are many in number and extend along the entire surface of the plasma membrane. They are short, hair-like structures that are used to move entire cells (such as paramecium) or move substances along the outer surface of the cell (for example, the cilia of cells lining the fallopian tubes that move the ovum toward the uterus, or cilia lining the cells of the respiratory tract that move particulate matter toward the throat that mucus has trapped).

The Endomembrane System

The endomembrane system (*endo* = within) is a group of membranes and organelles (Figure **1.1.3.3**) in eukaryotic cells that work together to modify, package, and transport lipids and proteins. It includes the nuclear envelope, lysosomes, and vesicles, the endoplasmic reticulum and Golgi apparatus, which we will cover shortly. Although not technically *within* the cell, the plasma membrane is included in the endomembrane system because, as you will see, it interacts with the other endomembranous organelles.

The Nucleus

Typically, the nucleus is the most prominent organelle in a cell (Figure **1.1.3.1**). The nucleus (plural = nuclei) houses the cell's DNA in the form of chromatin and directs the synthesis of ribosomes and proteins. Let us look at it in more detail (Figure **1.1.3.4**).

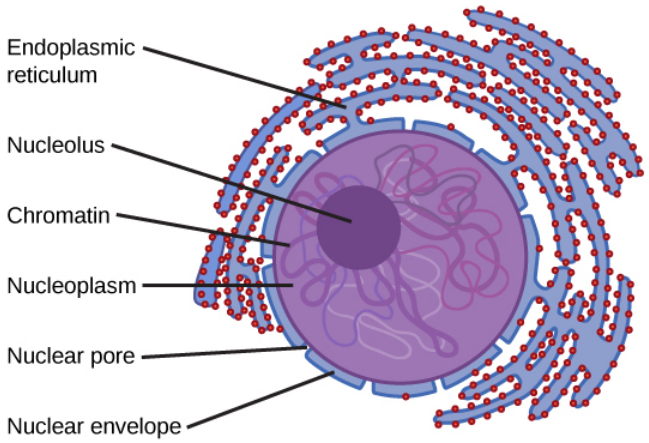


Figure 1.1.3.4: The outermost boundary of the nucleus is the nuclear envelope. Notice that the nuclear envelope consists of two phospholipid bilayers (membranes)—an outer membrane and an inner membrane—in contrast to the plasma membrane (Figure **1.1.3.2**), which consists of only one phospholipid bilayer. (credit: modification of work by NIGMS, NIH)

The nuclear envelope is a double-membrane structure that constitutes the outermost portion of the nucleus (Figure **1.1.3.4**). Both the inner and outer membranes of the nuclear envelope are phospholipid bilayers.

The nuclear envelope is punctuated with pores that control the passage of ions, molecules, and RNA between the nucleoplasm and the cytoplasm.

To understand chromatin, it is helpful to first consider chromosomes. Chromosomes are structures within the nucleus that are made up of DNA, the hereditary material, and proteins. This combination of DNA and proteins is called chromatin. In eukaryotes, chromosomes are linear structures. Every species has a specific number of chromosomes in the nucleus of its body cells. For example, in humans, the chromosome number is 46, whereas in fruit flies, the chromosome number is eight.

Chromosomes are only visible and distinguishable from one another when the cell is getting ready to divide. When the cell is in the growth and maintenance phases of its life cycle, the chromosomes resemble an unwound, jumbled bunch of threads.

We already know that the nucleus directs the synthesis of ribosomes, but how does it do this? Some chromosomes have sections of DNA that encode ribosomal RNA. A darkly staining area within the nucleus, called the nucleolus (plural = nucleoli), aggregates the ribosomal RNA with associated proteins to assemble the ribosomal subunits that are then transported through the nuclear pores into the cytoplasm.

The Endoplasmic Reticulum

The endoplasmic reticulum (ER) (Figure **1.1.3.7**) is a series of interconnected membranous tubules that collectively modify proteins and synthesize lipids. However, these two functions are performed in separate areas of the endoplasmic reticulum: the rough endoplasmic reticulum and the smooth endoplasmic reticulum, respectively.

The hollow portion of the ER tubules is called the lumen or cisternal space. The membrane of the ER, which is a phospholipid bilayer embedded with proteins, is continuous with the nuclear envelope.

The rough endoplasmic reticulum (RER) is so named because the ribosomes attached to its cytoplasmic surface give it a studded appearance when viewed through an electron microscope.

The ribosomes synthesize proteins while attached to the ER, resulting in transfer of their newly synthesized proteins into the lumen of the RER where they undergo modifications such as folding or addition of sugars. The RER also makes phospholipids for cell membranes.

If the phospholipids or modified proteins are not destined to stay in the RER, they will be packaged within vesicles and transported from the RER by budding from the membrane (Figure **1.1.3.7**). Since the RER is engaged in modifying proteins that will be secreted from the cell, it is abundant in cells that secrete proteins, such as the liver.

The smooth endoplasmic reticulum (SER) is continuous with the RER but has few or no ribosomes on its cytoplasmic surface (see Figure **1.1.3.1**). The SER's functions include synthesis of carbohydrates, lipids (including phospholipids), and steroid hormones; detoxification of medications and poisons; alcohol metabolism; and storage of calcium ions.

The Golgi Apparatus

We have already mentioned that vesicles can bud from the ER, but where do the vesicles go? Before reaching their final destination, the lipids or proteins within the transport vesicles need to be sorted, packaged, and tagged so that they wind up in the right place. The sorting, tagging, packaging, and distribution of lipids and proteins take place in the Golgi apparatus (also called the Golgi body), a series of flattened membranous sacs (Figure **1.1.3.5**).

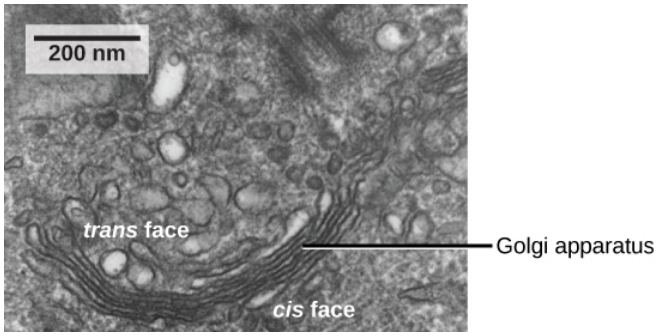


Figure 1.1.3.5: The Golgi apparatus in this transmission electron micrograph of a white blood cell is visible as a stack of semicircular flattened rings in the lower portion of this image. Several vesicles can be seen near the Golgi apparatus. (credit: modification of work by Louisa Howard; scale-bar data from Matt Russell)

The Golgi apparatus has a receiving face near the endoplasmic reticulum and a releasing face on the side away from the ER, toward the cell membrane. The transport vesicles that form from the ER travel to the receiving face, fuse with it, and empty their contents into the lumen of the Golgi apparatus. As the proteins and lipids travel through the Golgi, they undergo further modifications. The most frequent modification is the addition of short chains of sugar molecules. The newly modified proteins and lipids are then tagged with small molecular groups to enable them to be routed to their proper destinations.

Finally, the modified and tagged proteins are packaged into vesicles that bud from the opposite face of the Golgi. While some of these vesicles, transport vesicles, deposit their contents into other parts of the cell where they will be used, others, secretory vesicles, fuse with the plasma membrane and release their contents outside the cell.

The amount of Golgi in different cell types again illustrates that form follows function within cells. Cells that engage in a great deal of secretory activity (such as cells of the salivary glands that secrete digestive enzymes or cells of the immune system that secrete antibodies) have an abundant number of Golgi.

In plant cells, the Golgi has an additional role of synthesizing polysaccharides, some of which are incorporated into the cell wall and some of which are used in other parts of the cell.

Lysosomes

In animal cells, the lysosomes are the cell's "garbage disposal." Digestive enzymes within the lysosomes aid the breakdown of proteins, polysaccharides, lipids, nucleic acids, and even worn-out organelles. In single-celled eukaryotes, lysosomes are important for digestion of the food they ingest and the recycling of organelles. These enzymes are active at a much lower pH (more acidic)

than those located in the cytoplasm. Many reactions that take place in the cytoplasm could not occur at a low pH, thus the advantage of compartmentalizing the eukaryotic cell into organelles is apparent.

Lysosomes also use their hydrolytic enzymes to destroy disease-causing organisms that might enter the cell. A good example of this occurs in a group of white blood cells called macrophages, which are part of your body's immune system. In a process known as phagocytosis, a section of the plasma membrane of the macrophage invaginates (folds in) and engulfs a pathogen. The invaginated section, with the pathogen inside, then pinches itself off from the plasma membrane and becomes a vesicle. The vesicle fuses with a lysosome. The lysosome's hydrolytic enzymes then destroy the pathogen (Figure **1.1.3.6**).

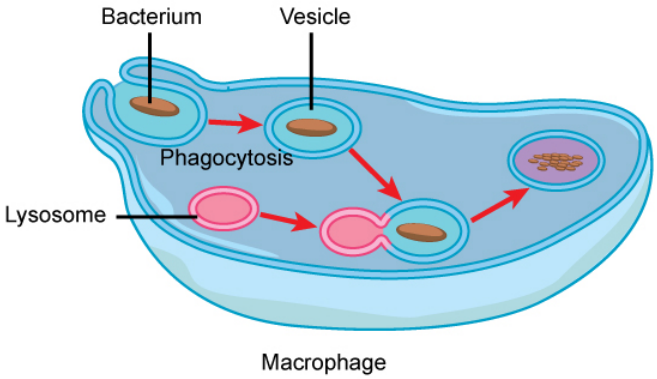


Figure 1.1.3.6: A macrophage has phagocytized a potentially pathogenic bacterium into a vesicle, which then fuses with a lysosome within the cell so that the pathogen can be destroyed. Other organelles are present in the cell, but for simplicity, are not shown.

Vesicles and Vacuoles

Vesicles and vacuoles are membrane-bound sacs that function in storage and transport. Vacuoles are somewhat larger than vesicles, and the membrane of a vacuole does not fuse with the membranes of other cellular components. Vesicles can fuse with other membranes within the cell system. Additionally, enzymes within plant vacuoles can break down macromolecules.

ART CONNECTION

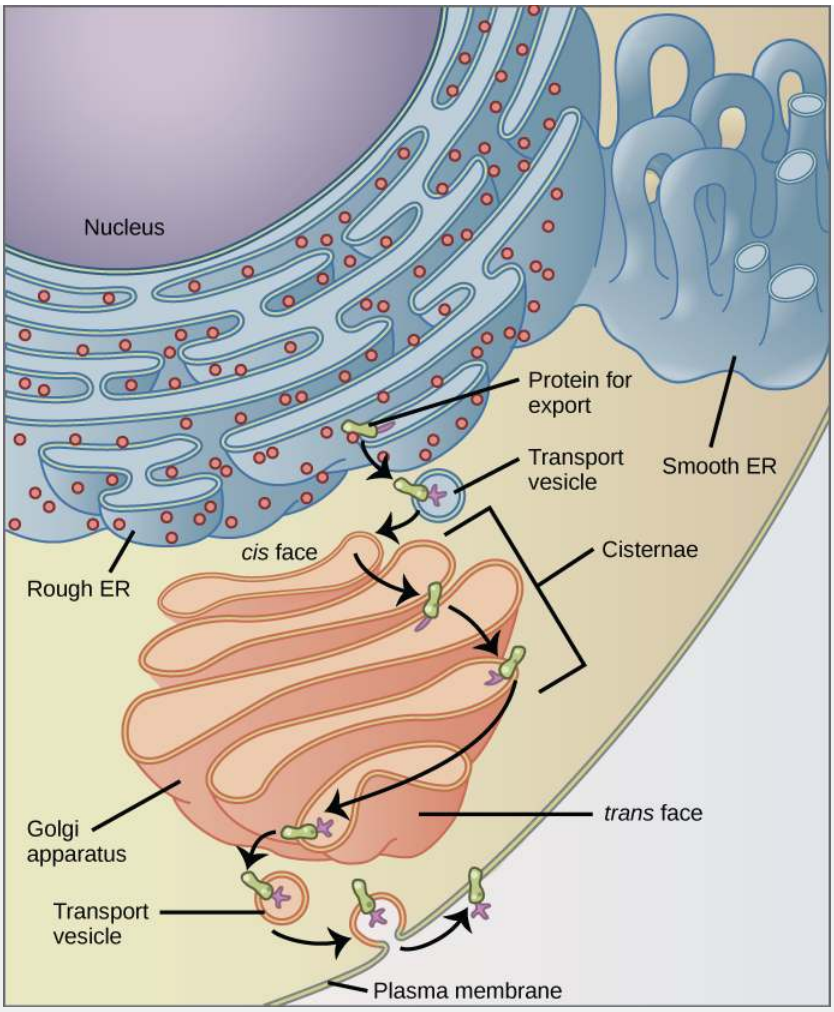


Figure 1.1.3.7: The endomembrane system works to modify, package, and transport lipids and proteins. (credit: modification of work by Magnus Manske)

Why does the *cis* face of the Golgi not face the plasma membrane?

Ribosomes

Ribosomes are the cellular structures responsible for protein synthesis. When viewed through an electron microscope, free ribosomes appear as either clusters or single tiny dots floating freely in the cytoplasm. Ribosomes may be attached to either the cytoplasmic side of the plasma membrane or the cytoplasmic side of the endoplasmic reticulum (Figure **1.1.3.7**). Electron microscopy has shown that ribosomes consist of large and small subunits. Ribosomes are enzyme complexes that are responsible for protein synthesis.

Because protein synthesis is essential for all cells, ribosomes are found in practically every cell, although they are smaller in prokaryotic cells. They are particularly abundant in immature red blood cells for the synthesis of hemoglobin, which functions in the transport of oxygen throughout the body.

Mitochondria

Mitochondria (singular = mitochondrion) are often called the “powerhouses” or “energy factories” of a cell because they are responsible for making adenosine triphosphate (ATP), the cell’s main energy-carrying molecule. The formation of ATP from the breakdown of glucose is known as cellular respiration. Mitochondria are oval-shaped, double-membrane organelles (Figure **1.1.3.8**) that have their own ribosomes and DNA. Each membrane is a phospholipid bilayer embedded with proteins. The inner layer has folds called cristae, which increase the surface area of the inner membrane. The area surrounded by the folds is called the mitochondrial matrix. The cristae and the matrix have different roles in cellular respiration.

In keeping with our theme of form following function, it is important to point out that muscle cells have a very high concentration of mitochondria because muscle cells need a lot of energy to contract.

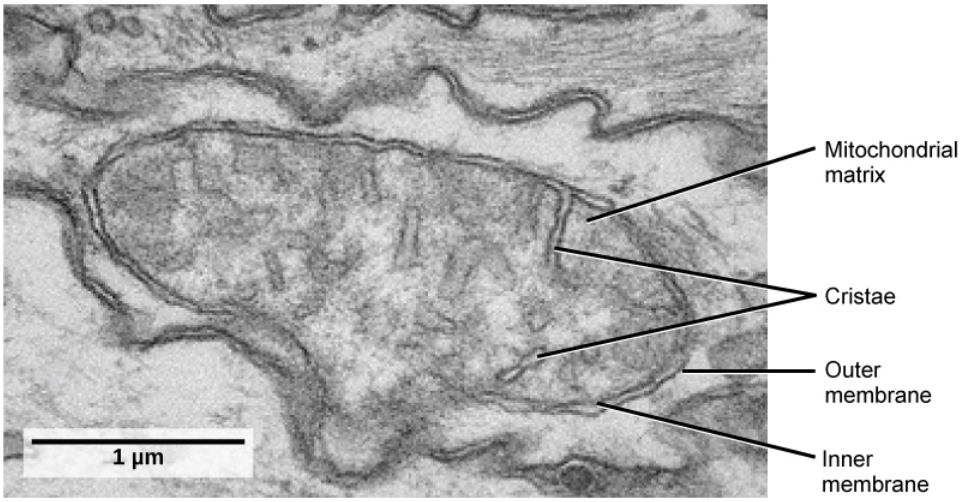


Figure 1.1.3.8: This transmission electron micrograph shows a mitochondrion as viewed with an electron microscope. Notice the inner and outer membranes, the cristae, and the mitochondrial matrix. (credit: modification of work by Matthew Britton; scale-bar data from Matt Russell)

Peroxisomes

Peroxisomes are small, round organelles enclosed by single membranes. They carry out oxidation reactions that break down fatty acids and amino acids. They also detoxify many poisons that may enter the body. Alcohol is detoxified by peroxisomes in liver cells. A byproduct of these oxidation reactions is hydrogen peroxide, H_2O_2, which is contained within the peroxisomes to prevent the chemical from causing damage to cellular components outside of the organelle. Hydrogen peroxide is safely broken down by peroxisomal enzymes into water and oxygen.

Animal Cells versus Plant Cells

Despite their fundamental similarities, there are some striking differences between animal and plant cells (see Table **1.1.3.1**). Animal cells have centrioles, centrosomes (discussed under the cytoskeleton), and lysosomes, whereas plant cells do not. Plant cells have a cell wall, chloroplasts, plasmodesmata, and plastids used for storage, and a large central vacuole, whereas animal cells do not.

The Cell Wall

In Figure **1.1.3.1b**, the diagram of a plant cell, you see a structure external to the plasma membrane called the cell wall. The cell wall is a rigid covering that protects the cell, provides structural support, and gives shape to the cell. Fungal and protist cells also have cell walls.

While the chief component of prokaryotic cell walls is peptidoglycan, the major organic molecule in the plant cell wall is cellulose, a polysaccharide made up of long, straight chains of glucose units. When nutritional information refers to dietary fiber, it is referring to the cellulose content of food.

Chloroplasts

Like mitochondria, chloroplasts also have their own DNA and ribosomes. Chloroplasts function in photosynthesis and can be found in eukaryotic cells such as plants and algae. In photosynthesis, carbon dioxide, water, and light energy are used to make glucose and oxygen. This is the major difference between plants and animals: Plants (autotrophs) are able to make their own food, like glucose, whereas animals (heterotrophs) must rely on other organisms for their organic compounds or food source.

Like mitochondria, chloroplasts have outer and inner membranes, but within the space enclosed by a chloroplast's inner membrane is a set of interconnected and stacked, fluid-filled membrane sacs called thylakoids (Figure **1.1.3.9**). Each stack of thylakoids is called a granum (plural = grana). The fluid enclosed by the inner membrane and surrounding the grana is called the stroma.

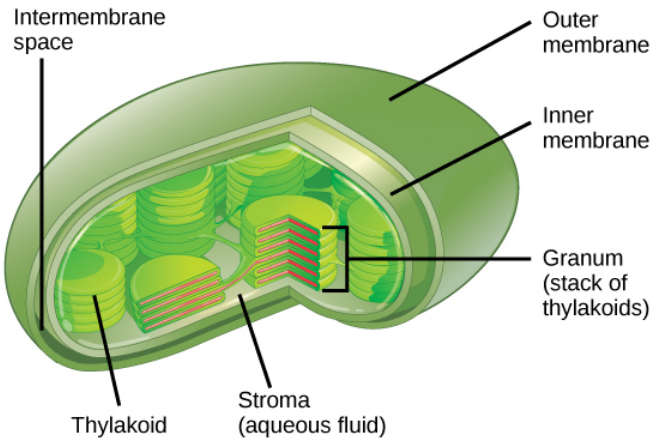


Figure 1.1.3.9: This simplified diagram of a chloroplast shows the outer membrane, inner membrane, thylakoids, grana, and stroma.

The chloroplasts contain a green pigment called chlorophyll, which captures the energy of sunlight for photosynthesis. Like plant cells, photosynthetic protists also have chloroplasts. Some bacteria also perform photosynthesis, but they do not have chloroplasts. Their photosynthetic pigments are located in the thylakoid membrane within the cell itself.

EVOLUTION IN ACTION: Endosymbiosis

We have mentioned that both mitochondria and chloroplasts contain DNA and ribosomes. Have you wondered why? Strong evidence points to endosymbiosis as the explanation.

Symbiosis is a relationship in which organisms from two separate species live in close association and typically exhibit specific adaptations to each other. Endosymbiosis (*endo-*= within) is a relationship in which one organism lives inside the other. Endosymbiotic relationships abound in nature. Microbes that produce vitamin K live inside the human gut. This relationship is beneficial for us because we are unable to synthesize vitamin K. It is also beneficial for the microbes because they are protected from other organisms and are provided a stable habitat and abundant food by living within the large intestine.

Scientists have long noticed that bacteria, mitochondria, and chloroplasts are similar in size. We also know that mitochondria and chloroplasts have DNA and ribosomes, just as bacteria do. Scientists believe that host cells and bacteria formed a mutually beneficial endosymbiotic relationship when the host cells ingested aerobic bacteria and cyanobacteria but did not destroy them. Through evolution, these ingested bacteria became more specialized in their functions, with the aerobic bacteria becoming mitochondria and the photosynthetic bacteria becoming chloroplasts.

The Central Vacuole

Previously, we mentioned vacuoles as essential components of plant cells. If you look at Figure **1.1.3.1**, you will see that plant cells each have a large, central vacuole that occupies most of the cell. The central vacuole plays a key role in regulating the cell's concentration of water in changing environmental conditions. In plant cells, the liquid inside the central vacuole provides turgor pressure, which is the outward pressure caused by the fluid inside the cell. Have you ever noticed that if you forget to water a plant for a few days, it wilts? That is because as the water concentration in the soil becomes lower than the water concentration in the plant, water moves out of the central vacuoles and cytoplasm and into the soil. As the central vacuole shrinks, it leaves the cell wall unsupported. This loss of support to the cell walls of a plant results in the wilted appearance. Additionally, this fluid has a very bitter taste, which discourages consumption by insects and animals. The central vacuole also functions to store proteins in developing seed cells.

Extracellular Matrix of Animal Cells

Most animal cells release materials into the extracellular space. The primary components of these materials are glycoproteins and the protein collagen. Collectively, these materials are called the extracellular matrix (Figure **1.1.3.10**). Not only does the extracellular matrix hold the cells together to form a tissue, but it also allows the cells within the tissue to communicate with each other.

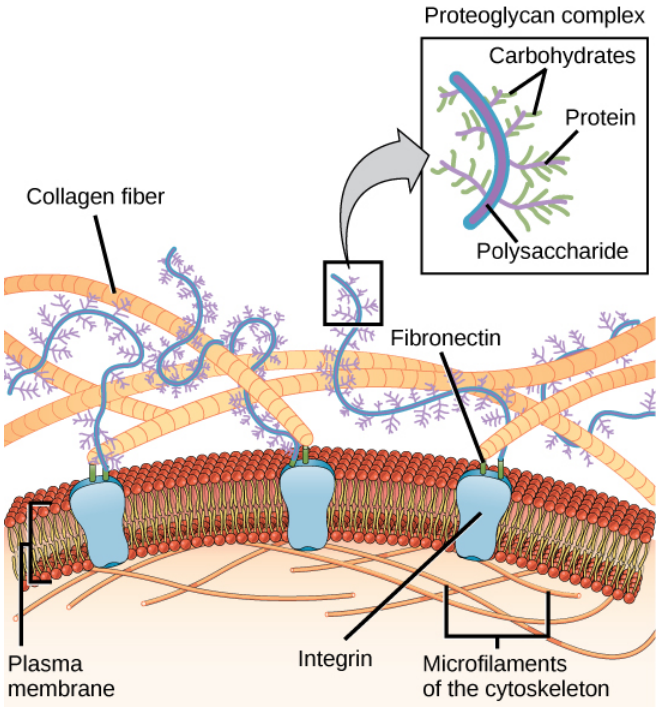


Figure 1.1.3.10: The extracellular matrix consists of a network of substances secreted by cells.

Blood clotting provides an example of the role of the extracellular matrix in cell communication. When the cells lining a blood vessel are damaged, they display a protein receptor called tissue factor. When tissue factor binds with another factor in the extracellular matrix, it causes platelets to adhere to the wall of the damaged blood vessel, stimulates adjacent smooth muscle cells in the blood vessel to contract (thus constricting the blood vessel), and initiates a series of steps that stimulate the platelets to produce clotting factors.

Intercellular Junctions

Cells can also communicate with each other by direct contact, referred to as intercellular junctions. There are some differences in the ways that plant and animal cells do this. Plasmodesmata (singular = plasmodesma) are junctions between plant cells, whereas animal cell contacts include tight and gap junctions, and desmosomes.

In general, long stretches of the plasma membranes of neighboring plant cells cannot touch one another because they are separated by the cell walls surrounding each cell. Plasmodesmata are numerous channels that pass between the cell walls of adjacent plant cells, connecting their cytoplasm and enabling signal molecules and nutrients to be transported from cell to cell (Figure **1.1.3.11a**).

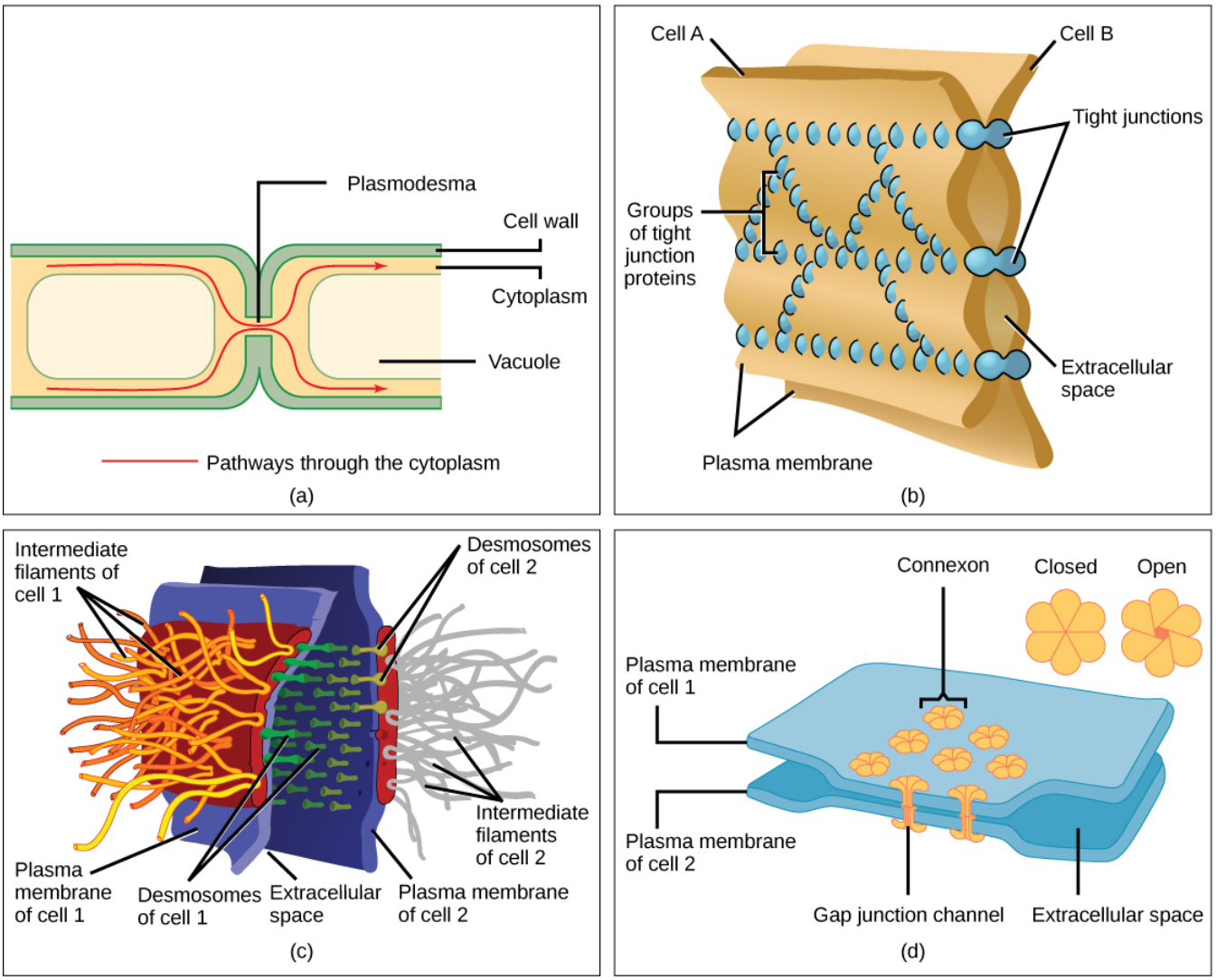


Figure 1.1.3.11: There are four kinds of connections between cells. (a) A plasmodesma is a channel between the cell walls of two adjacent plant cells. (b) Tight junctions join adjacent animal cells. (c) Desmosomes join two animal cells together. (d) Gap junctions act as channels between animal cells. (credit b, c, d: modification of work by Mariana Ruiz Villareal)

A tight junction is a watertight seal between two adjacent animal cells (Figure **1.1.3.11b**). Proteins hold the cells tightly against each other. This tight adhesion prevents materials from leaking between the cells. Tight junctions are typically found in the epithelial tissue that lines internal organs and cavities, and composes most of the skin. For example, the tight junctions of the epithelial cells lining the urinary bladder prevent urine from leaking into the extracellular space.

Also found only in animal cells are desmosomes, which act like spot welds between adjacent epithelial cells (Figure **1.1.3.11c**). They keep cells together in a sheet-like formation in organs and tissues that stretch, like the skin, heart, and muscles.

Gap junctions in animal cells are like plasmodesmata in plant cells in that they are channels between adjacent cells that allow for the transport of ions, nutrients, and other substances that enable cells to communicate (Figure **1.1.3.11d**). Structurally, however, gap junctions and plasmodesmata differ.

Table 1.1.3.1: This table provides the components of prokaryotic and eukaryotic cells and their respective functions.

Cell Component	Function	Present in Prokaryotes?	Present in Animal Cells?	Present in Plant Cells?
Plasma membrane	Separates cell from external environment; controls passage of organic molecules, ions, water, oxygen, and wastes into and out of the cell	Yes	Yes	Yes
Cytoplasm	Provides structure to cell; site of many metabolic reactions; medium in which organelles are found	Yes	Yes	Yes
Nucleoid	Location of DNA	Yes	No	No
Nucleus	Cell organelle that houses DNA and directs synthesis of ribosomes and proteins	No	Yes	Yes
Ribosomes	Protein synthesis	Yes	Yes	Yes
Mitochondria	ATP production/cellular respiration	No	Yes	Yes
Peroxisomes	Oxidizes and breaks down fatty acids and amino acids, and detoxifies poisons	No	Yes	Yes
Vesicles and vacuoles	Storage and transport; digestive function in plant cells	No	Yes	Yes
Centrosome	Unspecified role in cell division in animal cells; organizing center of microtubules in animal cells	No	Yes	No
Lysosomes	Digestion of macromolecules; recycling of worn-out organelles	No	Yes	No
Cell wall	Protection, structural support and maintenance of cell shape	Yes, primarily peptidoglycan in bacteria but not Archaea	No	Yes, primarily cellulose
Chloroplasts	Photosynthesis	No	No	Yes
Endoplasmic reticulum	Modifies proteins and synthesizes lipids	No	Yes	Yes
Golgi apparatus	Modifies, sorts, tags,	No	Yes	Yes

Cell Component	Function	Present in Prokaryotes?	Present in Animal Cells?	Present in Plant Cells?
	packages, and distributes lipids and proteins			
Cytoskeleton	Maintains cell's shape, secures organelles in specific positions, allows cytoplasm and vesicles to move within the cell, and enables unicellular organisms to move independently	Yes	Yes	Yes
Flagella	Cellular locomotion	Some	Some	No, except for some plant sperm.
Cilia	Cellular locomotion, movement of particles along extracellular surface of plasma membrane, and filtration	No	Some	No

Summary

Like a prokaryotic cell, a eukaryotic cell has a plasma membrane, cytoplasm, and ribosomes, but a eukaryotic cell is typically larger than a prokaryotic cell, has a true nucleus (meaning its DNA is surrounded by a membrane), and has other membrane-bound organelles that allow for compartmentalization of functions. The plasma membrane is a phospholipid bilayer embedded with proteins. The nucleolus within the nucleus is the site for ribosome assembly. Ribosomes are found in the cytoplasm or are attached to the cytoplasmic side of the plasma membrane or endoplasmic reticulum. They perform protein synthesis. Mitochondria perform cellular respiration and produce ATP. Peroxisomes break down fatty acids, amino acids, and some toxins. Vesicles and vacuoles are storage and transport compartments. In plant cells, vacuoles also help break down macromolecules.

Animal cells also have a centrosome and lysosomes. The centrosome has two bodies, the centrioles, with an unknown role in cell division. Lysosomes are the digestive organelles of animal cells.

Plant cells have a cell wall, chloroplasts, and a central vacuole. The plant cell wall, whose primary component is cellulose, protects the cell, provides structural support, and gives shape to the cell. Photosynthesis takes place in chloroplasts. The central vacuole expands, enlarging the cell without the need to produce more cytoplasm.

The endomembrane system includes the nuclear envelope, the endoplasmic reticulum, Golgi apparatus, lysosomes, vesicles, as well as the plasma membrane. These cellular components work together to modify, package, tag, and transport membrane lipids and proteins.

The cytoskeleton has three different types of protein elements. Microfilaments provide rigidity and shape to the cell, and facilitate cellular movements. Intermediate filaments bear tension and anchor the nucleus and other organelles in place. Microtubules help the cell resist compression, serve as tracks for motor proteins that move vesicles through the cell, and pull replicated chromosomes to opposite ends of a dividing cell. They are also the structural elements of centrioles, flagella, and cilia.

Animal cells communicate through their extracellular matrices and are connected to each other by tight junctions, desmosomes, and gap junctions. Plant cells are connected and communicate with each other by plasmodesmata.

Art Connections

Figure 1.1.3.1 What structures does a plant cell have that an animal cell does not have? What structures does an animal cell have that a plant cell does not have?

Figure 1.1.3.1 Plant cells have plasmodesmata, a cell wall, a large central vacuole, chloroplasts, and plastids. Animal cells have lysosomes and centrosomes.

Figure 1.1.3.7 Why does the *cis* face of the Golgi not face the plasma membrane?

Figure 1.1.3.7 Because that face receives chemicals from the ER, which is toward the center of the cell.

Multiple Choice

Which of the following is found both in eukaryotic and prokaryotic cells?

A. nucleus
B. mitochondrion
C. vacuole
D. ribosome

D

Which of the following is not a component of the endomembrane system?

A. mitochondrion
B. Golgi apparatus
C. endoplasmic reticulum
D. lysosome

A

Free Response

In the context of cell biology, what do we mean by form follows function? What are at least two examples of this concept?

“Form follows function” refers to the idea that the function of a body part dictates the form of that body part. As an example, organisms like birds or fish that fly or swim quickly through the air or water have streamlined bodies that reduce drag. At the level of the cell, in tissues involved in secretory functions, such as the salivary glands, the cells have abundant Golgi.

Glossary

cell wall
a rigid cell covering made of cellulose in plants, peptidoglycan in bacteria, non-peptidoglycan compounds in Archaea, and chitin in fungi that protects the cell, provides structural support, and gives shape to the cell

central vacuole
a large plant cell organelle that acts as a storage compartment, water reservoir, and site of macromolecule degradation

chloroplast
a plant cell organelle that carries out photosynthesis

cilium
(plural: cilia) a short, hair-like structure that extends from the plasma membrane in large numbers and is used to move an entire cell or move substances along the outer surface of the cell

cytoplasm

the entire region between the plasma membrane and the nuclear envelope, consisting of organelles suspended in the gel-like cytosol, the cytoskeleton, and various chemicals

cytoskeleton

the network of protein fibers that collectively maintains the shape of the cell, secures some organelles in specific positions, allows cytoplasm and vesicles to move within the cell, and enables unicellular organisms to move

cytosol

the gel-like material of the cytoplasm in which cell structures are suspended

desmosome

a linkage between adjacent epithelial cells that forms when cadherins in the plasma membrane attach to intermediate filaments

endomembrane system

the group of organelles and membranes in eukaryotic cells that work together to modify, package, and transport lipids and proteins

endoplasmic reticulum (ER)

a series of interconnected membranous structures within eukaryotic cells that collectively modify proteins and synthesize lipids

extracellular matrix

the material, primarily collagen, glycoproteins, and proteoglycans, secreted from animal cells that holds cells together as a tissue, allows cells to communicate with each other, and provides mechanical protection and anchoring for cells in the tissue

flagellum

(plural: flagella) the long, hair-like structure that extends from the plasma membrane and is used to move the cell

gap junction

a channel between two adjacent animal cells that allows ions, nutrients, and other low-molecular weight substances to pass between the cells, enabling the cells to communicate

Golgi apparatus

a eukaryotic organelle made up of a series of stacked membranes that sorts, tags, and packages lipids and proteins for distribution

lysosome

an organelle in an animal cell that functions as the cell's digestive component; it breaks down proteins, polysaccharides, lipids, nucleic acids, and even worn-out organelles

mitochondria

(singular: mitochondrion) the cellular organelles responsible for carrying out cellular respiration, resulting in the production of ATP, the cell's main energy-carrying molecule

nuclear envelope

the double-membrane structure that constitutes the outermost portion of the nucleus

nucleolus

the darkly staining body within the nucleus that is responsible for assembling ribosomal subunits

nucleus

the cell organelle that houses the cell's DNA and directs the synthesis of ribosomes and proteins

peroxisome

a small, round organelle that contains hydrogen peroxide, oxidizes fatty acids and amino acids, and detoxifies many poisons

plasma membrane
a phospholipid bilayer with embedded (integral) or attached (peripheral) proteins that separates the internal contents of the cell from its surrounding environment

plasmodesma
(plural: plasmodesmata) a channel that passes between the cell walls of adjacent plant cells, connects their cytoplasm, and allows materials to be transported from cell to cell

ribosome
a cellular structure that carries out protein synthesis

rough endoplasmic reticulum (RER)
the region of the endoplasmic reticulum that is studded with ribosomes and engages in protein modification

smooth endoplasmic reticulum (SER)
the region of the endoplasmic reticulum that has few or no ribosomes on its cytoplasmic surface and synthesizes carbohydrates, lipids, and steroid hormones; detoxifies chemicals like pesticides, preservatives, medications, and environmental pollutants, and stores calcium ions

tight junction
a firm seal between two adjacent animal cells created by protein adherence

vacuole
a membrane-bound sac, somewhat larger than a vesicle, that functions in cellular storage and transport

vesicle
a small, membrane-bound sac that functions in cellular storage and transport; its membrane is capable of fusing with the plasma membrane and the membranes of the endoplasmic reticulum and Golgi apparatus

Contributors and Attributions

- Template:ContribOpenStaxConcepts

This page titled 1.1.3: Eukaryotic Cells is shared under a CC BY license and was authored, remixed, and/or curated by .

- **3.3: Eukaryotic Cells** by OpenStax is licensed CC BY 4.0.

1.2: Atoms, Ions, Molecules

Skills to Develop

- Define matter and elements
- Describe the interrelationship between protons, neutrons, and electrons
- Compare the ways in which electrons can be donated or shared between atoms
- Explain the ways in which naturally occurring elements combine to create molecules, cells, tissues, organ systems, and organisms

At its most fundamental level, life is made up of matter. Matter is any substance that occupies space and has mass. Elements are unique forms of matter with specific chemical and physical properties that cannot be broken down into smaller substances by ordinary chemical reactions. There are 118 elements, but only 92 occur naturally. The remaining elements are synthesized in laboratories and are unstable.

Each element is designated by its chemical symbol, which is a single capital letter or, when the first letter is already "taken" by another element, a combination of two letters. Some elements follow the English term for the element, such as C for carbon and Ca for calcium. Other elements' chemical symbols derive from their Latin names; for example, the symbol for sodium is Na, referring to *natrium*, the Latin word for sodium.

The four elements common to all living organisms are oxygen (O), carbon (C), hydrogen (H), and nitrogen (N). In the non-living world, elements are found in different proportions, and some elements common to living organisms are relatively rare on the earth as a whole, as shown in Table **1.2.1**. For example, the atmosphere is rich in nitrogen and oxygen but contains little carbon and hydrogen, while the earth's crust, although it contains oxygen and a small amount of hydrogen, has little nitrogen and carbon. In spite of their differences in abundance, all elements and the chemical reactions between them obey the same chemical and physical laws regardless of whether they are a part of the living or non-living world.

Table 1.2.1: Approximate Percentage of Elements in Living Organisms (Humans) Compared to the Non-living World

Element	Life (Humans)	Atmosphere	Earth's Crust
Oxygen (O)	65%	21%	46%
Carbon (C)	18%	trace	trace
Hydrogen (H)	10%	trace	0.1%
Nitrogen (N)	3%	78%	trace

The Structure of the Atom

To understand how elements come together, we must first discuss the smallest component or building block of an element, the atom. An atom is the smallest unit of matter that retains all of the chemical properties of an element. For example, one gold atom has all of the properties of gold in that it is a solid metal at room temperature. A gold coin is simply a very large number of gold atoms molded into the shape of a coin and containing small amounts of other elements known as impurities. Gold atoms cannot be broken down into anything smaller while still retaining the properties of gold.

An atom is composed of two regions: the nucleus, which is in the center of the atom and contains protons and neutrons, and the outermost region of the atom which holds its electrons in orbit around the nucleus, as illustrated in Figure **1.2.1**. Atoms contain protons, electrons, and neutrons, among other subatomic particles. The only exception is hydrogen (H), which is made of one proton and one electron with no neutrons.

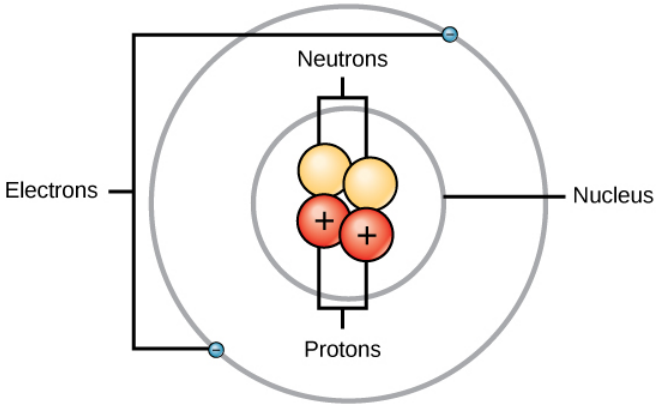


Figure **1.2.1:** Elements, such as helium, depicted here, are made up of atoms. Atoms are made up of protons and neutrons located within the nucleus, with electrons in orbitals surrounding the nucleus.

Protons and neutrons have approximately the same mass, about 1.67×10^{-24} grams. Scientists arbitrarily define this amount of mass as one atomic mass unit (amu) or one Dalton, as shown in Table **1.2.2**. Although similar in mass, protons and neutrons differ in their electric charge. A proton is positively charged whereas a neutron is uncharged. Therefore, the number of neutrons in an atom contributes significantly to its mass, but not to its charge. Electrons are much smaller in mass than protons, weighing only 9.11×10^{-28} grams, or about 1/1800 of an atomic mass unit. Hence, they do not contribute much to an element's overall atomic mass. Therefore, when considering atomic mass, it is customary to ignore the mass of any electrons and calculate the atom's mass based on the number of protons and neutrons alone. Although not significant contributors to mass, electrons do contribute greatly to the atom's charge, as each electron has a negative charge equal to the positive charge of a proton. In uncharged, neutral atoms, the number of electrons orbiting the nucleus is equal to the number of protons inside the nucleus. In these atoms, the positive and negative charges cancel each other out, leading to an atom with no net charge.

Accounting for the sizes of protons, neutrons, and electrons, most of the volume of an atom—greater than 99 percent—is, in fact, empty space. With all this empty space, one might ask why so-called solid objects do not just pass through one another. The reason they do not is that the electrons that surround all atoms are negatively charged and negative charges repel each other.

Table 1.2.2: Protons, Neutrons, and Electrons

	Charge	Mass (amu)	Location
Proton	+1	1	nucleus
Neutron	0	1	nucleus
Electron	–1	0	orbitals

Atomic Number and Mass

Atoms of each element contain a characteristic number of protons and electrons. The number of protons determines an element's atomic number and is used to distinguish one element from another. The number of neutrons is variable, resulting in isotopes, which are different forms of the same atom that vary only in the number of neutrons they possess. Together, the number of protons and the number of neutrons determine an element's mass number, as illustrated in Figure **1.2.2**. Note that the small contribution of mass from electrons is disregarded in calculating the mass number. This approximation of mass can be used to easily calculate how many neutrons an element has by simply subtracting the number of protons from the mass number. Since an element's isotopes will have slightly different mass numbers, scientists also determine the atomic mass, which is the calculated mean of the mass number for its naturally occurring isotopes. Often, the resulting number contains a fraction. For example, the atomic mass of chlorine (Cl) is 35.45 because chlorine is composed of several isotopes, some (the majority) with atomic mass 35 (17 protons and 18 neutrons) and some with atomic mass 37 (17 protons and 20 neutrons).

Art Connection

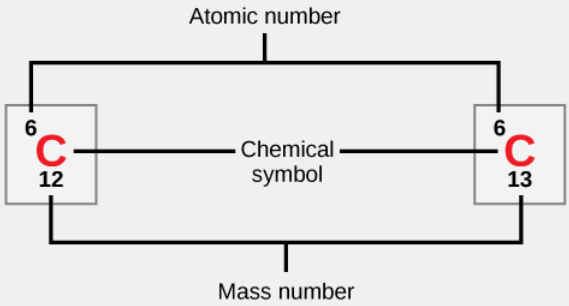


Figure **1.2.2**: Carbon has an atomic number of six, and two stable isotopes with mass numbers of twelve and thirteen, respectively. Its atomic mass is 12.11.

How many neutrons do carbon-12 and carbon-13 have, respectively?

Isotopes

Isotopes are different forms of an element that have the same number of protons but a different number of neutrons. Some elements—such as carbon, potassium, and uranium—have naturally occurring isotopes. Carbon-12 contains six protons, six neutrons, and six electrons; therefore, it has a mass number of 12 (six protons and six neutrons). Carbon-14 contains six protons, eight neutrons, and six electrons; its atomic mass is 14 (six protons and eight neutrons). These two alternate forms of carbon are isotopes. Some isotopes may emit neutrons, protons, and electrons, and attain a more stable atomic configuration (lower level of potential energy); these are radioactive isotopes, or radioisotopes. Radioactive decay (carbon-14 losing neutrons to eventually become carbon-12) describes the energy loss that occurs when an unstable atom's nucleus releases radiation.

Evolution Connection: Carbon Dating

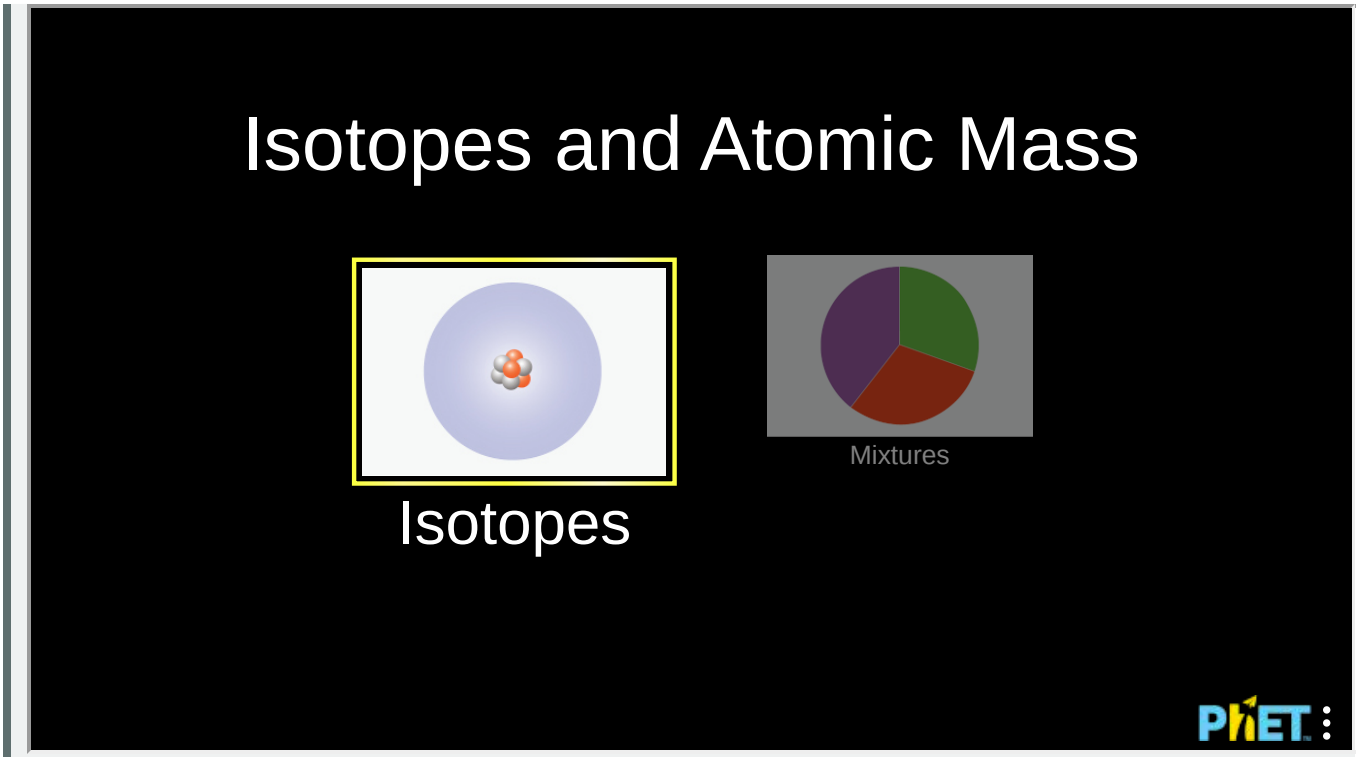
Carbon is normally present in the atmosphere in the form of gaseous compounds like carbon dioxide and methane. Carbon-14 (C^{14}) is a naturally occurring radioisotope that is created in the atmosphere from atmospheric N^{14} (nitrogen) by the addition of a neutron and the loss of a proton because of cosmic rays. This is a continuous process, so more C^{14} is always being created. As a living organism incorporates C^{14} initially as carbon dioxide fixed in the process of photosynthesis, the relative amount of C^{14} in its body is equal to the concentration of C^{14} in the atmosphere. When an organism dies, it is no longer ingesting C^{14}, so the ratio between C^{14} and C^{12} will decline as C^{14} decays gradually to N^{14} by a process called beta decay—the emission of electrons or positrons. This decay gives off energy in a slow process.

After approximately 5,730 years, half of the starting concentration of C^{14} will have been converted back to N^{14}. The time it takes for half of the original concentration of an isotope to decay back to its more stable form is called its half-life. Because the half-life of C^{14} is long, it is used to date formerly living objects such as old bones or wood. Comparing the ratio of the C^{14} concentration found in an object to the amount of C^{14} detected in the atmosphere, the amount of the isotope that has not yet decayed can be determined. On the basis of this amount, the age of the material, such as the pygmy mammoth shown in Figure **1.2.3**, can be calculated with accuracy if it is not much older than about 50,000 years. Other elements have isotopes with different half lives. For example, K^{40} (potassium-40) has a half-life of 1.25 billion years, and U^{235} (Uranium 235) has a half-life of about 700 million years. Through the use of radiometric dating, scientists can study the age of fossils or other remains of extinct organisms to understand how organisms have evolved from earlier species.



Figure **1.2.3**: The age of carbon-containing remains less than about 50,000 years old, such as this pygmy mammoth, can be determined using carbon dating. (credit: Bill Faulkner, NPS)

Link to Learning



Video: To learn more about atoms, isotopes, and how to tell one isotope from another, visit this site and run the simulation.

The Periodic Table

The different elements are organized and displayed in the periodic table. Devised by Russian chemist Dmitri Mendeleev (1834–1907) in 1869, the table groups elements that, although unique, share certain chemical properties with other elements. The properties of elements are responsible for their physical state at room temperature: they may be gases, solids, or liquids. Elements also have specific chemical reactivity, the ability to combine and to chemically bond with each other.

In the periodic table, shown in Figure **1.2.4**, the elements are organized and displayed according to their atomic number and are arranged in a series of rows and columns based on shared chemical and physical properties. In addition to providing the atomic number for each element, the periodic table also displays the element's atomic mass. Looking at carbon, for example, its symbol (C) and name appear, as well as its atomic number of six (in the upper left-hand corner) and its atomic mass of 12.11.

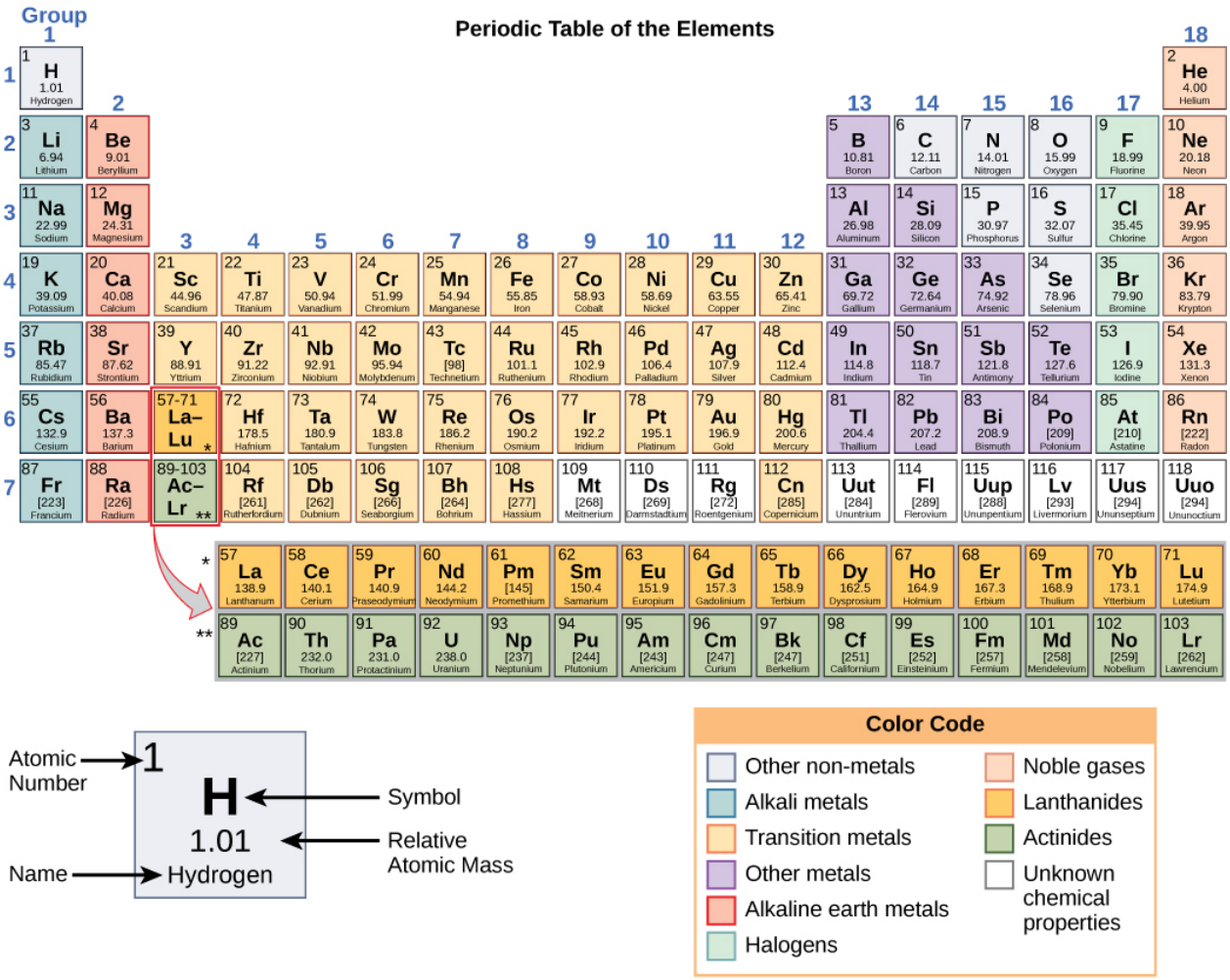


Figure **1.2.4**: The periodic table shows the atomic mass and atomic number of each element. The atomic number appears above the symbol for the element and the approximate atomic mass appears below it.

The periodic table groups elements according to chemical properties. The differences in chemical reactivity between the elements are based on the number and spatial distribution of an atom's electrons. Atoms that chemically react and bond to each other form molecules. Molecules are simply two or more atoms chemically bonded together. Logically, when two atoms chemically bond to form a molecule, their electrons, which form the outermost region of each atom, come together first as the atoms form a chemical bond.

Electron Shells and the Bohr Model

It should be stressed that there is a connection between the number of protons in an element, the atomic number that distinguishes one element from another, and the number of electrons it has. In all electrically neutral atoms, the number of electrons is the same as the number of protons. Thus, each element, at least when electrically neutral, has a characteristic number of electrons equal to its atomic number.

An early model of the atom was developed in 1913 by Danish scientist Niels Bohr (1885–1962). The Bohr model shows the atom as a central nucleus containing protons and neutrons, with the electrons in circular orbitals at specific distances from the nucleus, as illustrated in Figure **1.2.5**. These orbits form electron shells or energy levels, which are a way of visualizing the number of electrons in the outermost shells. These energy levels are designated by a number and the symbol "n." For example, 1n represents the first energy level located closest to the nucleus.

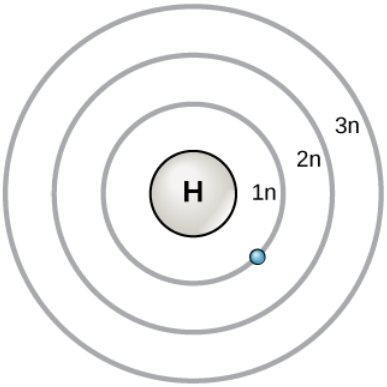


Figure **1.2.5**: The Bohr model was developed by Niels Bohrs in 1913. In this model, electrons exist within principal shells. An electron normally exists in the lowest energy shell available, which is the one closest to the nucleus. Energy from a photon of light can bump it up to a higher energy shell, but this situation is unstable, and the electron quickly decays back to the ground state. In the process, a photon of light is released

Electrons fill orbitals in a consistent order: they first fill the orbitals closest to the nucleus, then they continue to fill orbitals of increasing energy further from the nucleus. If there are multiple orbitals of equal energy, they will be filled with one electron in each energy level before a second electron is added. The electrons of the outermost energy level determine the energetic stability of the atom and its tendency to form chemical bonds with other atoms to form molecules.

Under standard conditions, atoms fill the inner shells first, often resulting in a variable number of electrons in the outermost shell. The innermost shell has a maximum of two electrons but the next two electron shells can each have a maximum of eight electrons. This is known as the octet rule, which states, with the exception of the innermost shell, that atoms are more stable energetically when they have eight electrons in their valence shell, the outermost electron shell. Examples of some neutral atoms and their electron configurations are shown in Figure **1.2.6**. Notice that in this figure, helium has a complete outer electron shell, with two electrons filling its first and only shell. Similarly, neon has a complete outer 2n shell containing eight electrons. In contrast, chlorine and sodium have seven and one in their outer shells, respectively, but theoretically they would be more energetically stable if they followed the octet rule and had eight.

Art Connection

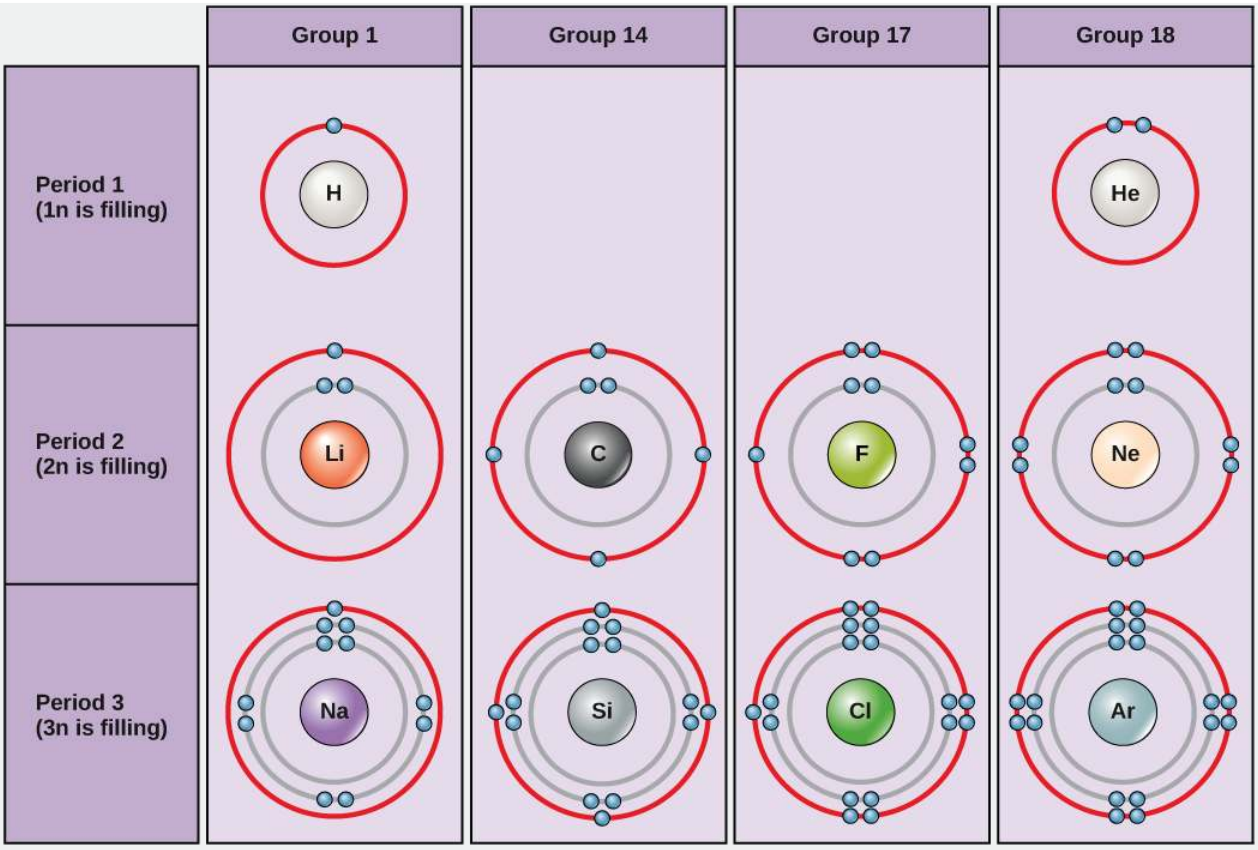


Figure **1.2.6**: Bohr diagrams indicate how many electrons fill each principal shell. Group 18 elements (helium, neon, and argon are shown) have a full outer, or valence, shell. A full valence shell is the most stable electron configuration. Elements in other groups have partially filled valence shells and gain or lose electrons to achieve a stable electron configuration.

An atom may give, take, or share electrons with another atom to achieve a full valence shell, the most stable electron configuration. Looking at this figure, how many electrons do elements in group 1 need to lose in order to achieve a stable electron configuration? How many electrons do elements in groups 14 and 17 need to gain to achieve a stable configuration?

Understanding that the organization of the periodic table is based on the total number of protons (and electrons) helps us know how electrons are distributed among the outer shell. The periodic table is arranged in columns and rows based on the number of electrons and where these electrons are located. Take a closer look at the some of the elements in the table's far right column in Figure **1.2.6**. The group 18 atoms helium (He), neon (Ne), and argon (Ar) all have filled outer electron shells, making it unnecessary for them to share electrons with other atoms to attain stability; they are highly stable as single atoms. Their non-reactivity has resulted in their being named the inert gases (or noble gases). Compare this to the group 1 elements in the left-hand column. These elements, including hydrogen (H), lithium (Li), and sodium (Na), all have one electron in their outermost shells. That means that they can achieve a stable configuration and a filled outer shell by donating or sharing one electron with another atom or a molecule such as water. Hydrogen will donate or share its electron to achieve this configuration, while lithium and sodium will donate their electron to become stable. As a result of losing a negatively charged electron, they become positively charged ions. Group 17 elements, including fluorine and chlorine, have seven electrons in their outmost shells, so they tend to fill this shell with an electron from other atoms or molecules, making them negatively charged ions. Group 14 elements, of which carbon is the most important to living systems, have four electrons in their outer shell allowing them to make several covalent bonds (discussed below) with other atoms. Thus, the columns of the periodic table represent the potential shared state of these elements' outer electron shells that is responsible for their similar chemical characteristics.

Electron Orbitals

Although useful to explain the reactivity and chemical bonding of certain elements, the Bohr model of the atom does not accurately reflect how electrons are spatially distributed surrounding the nucleus. They do not circle the nucleus like the earth orbits the sun,

but are found in electron orbitals. These relatively complex shapes result from the fact that electrons behave not just like particles, but also like waves. Mathematical equations from quantum mechanics known as wave functions can predict within a certain level of probability where an electron might be at any given time. The area where an electron is most likely to be found is called its orbital.

Recall that the Bohr model depicts an atom's electron shell configuration. Within each electron shell are subshells, and each subshell has a specified number of orbitals containing electrons. While it is impossible to calculate exactly where an electron is located, scientists know that it is most probably located within its orbital path. Subshells are designated by the letter s, *p*, *d*, and *f*. The *s* subshell is spherical in shape and has one orbital. Principal shell 1n has only a single *s* orbital, which can hold two electrons. Principal shell 2n has one *s* and one *p* subshell, and can hold a total of eight electrons. The *p* subshell has three dumbbell-shaped orbitals, as illustrated in Figure **1.2.7**. Subshells *d* and *f* have more complex shapes and contain five and seven orbitals, respectively. These are not shown in the illustration. Principal shell 3n has *s*, *p*, and *d* subshells and can hold 18 electrons. Principal shell 4n has *s*, *p*, *d* and *f* orbitals and can hold 32 electrons. Moving away from the nucleus, the number of electrons and orbitals found in the energy levels increases. Progressing from one atom to the next in the periodic table, the electron structure can be worked out by fitting an extra electron into the next available orbital.

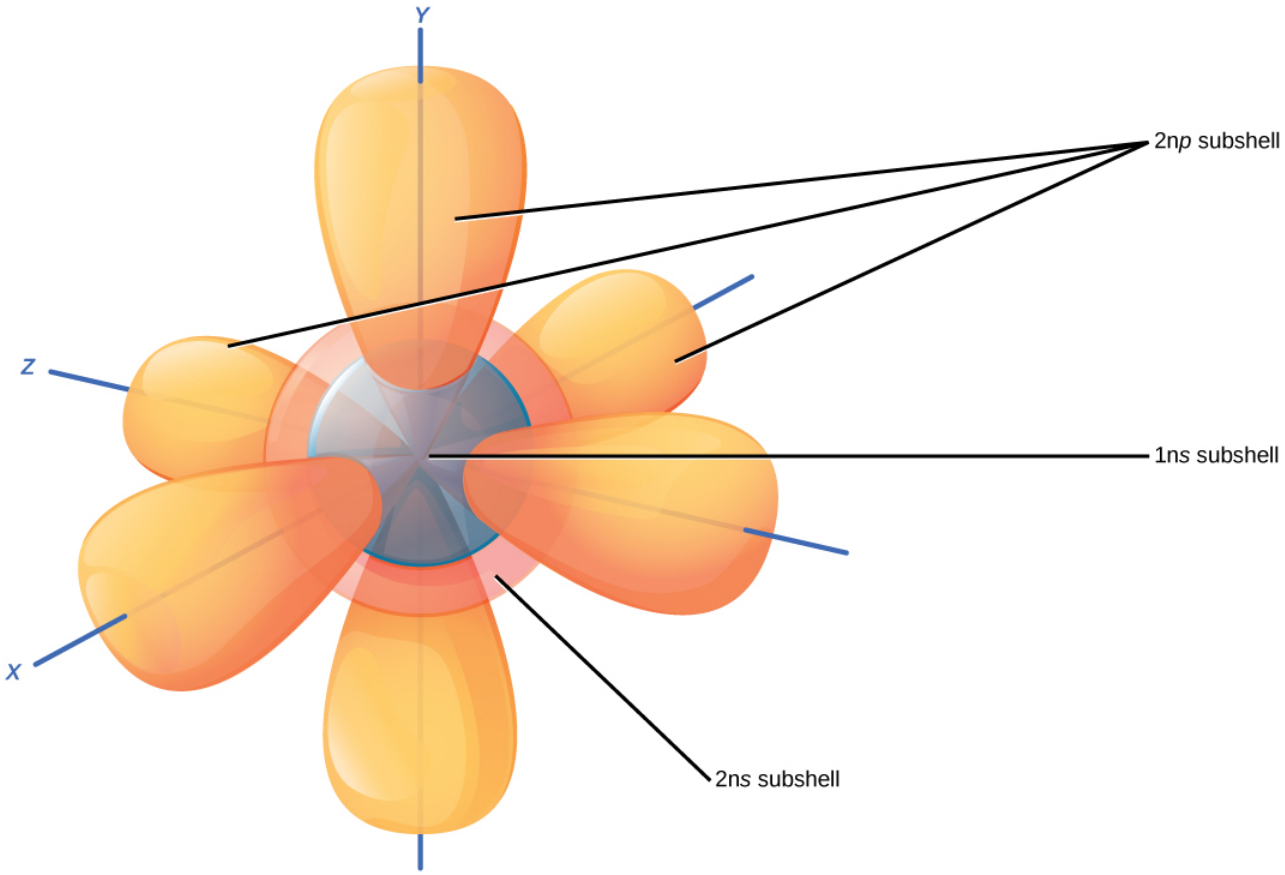


Figure **1.2.7:** The s subshells are shaped like spheres. Both the 1n and 2n principal shells have an s orbital, but the size of the sphere is larger in the 2n orbital. Each sphere is a single orbital. p subshells are made up of three dumbbell-shaped orbitals. Principal shell 2n has a p subshell, but shell 1 does not.

The closest orbital to the nucleus, called the 1s orbital, can hold up to two electrons. This orbital is equivalent to the innermost electron shell of the Bohr model of the atom. It is called the 1*s* orbital because it is spherical around the nucleus. The 1*s* orbital is the closest orbital to the nucleus, and it is always filled first, before any other orbital can be filled. Hydrogen has one electron; therefore, it has only one spot within the 1*s* orbital occupied. This is designated as $1s^1$, where the superscripted 1 refers to the one electron within the 1*s* orbital. Helium has two electrons; therefore, it can completely fill the 1*s* orbital with its two electrons. This is designated as $1s^2$, referring to the two electrons of helium in the 1*s* orbital. On the periodic table Figure **1.2.4**, hydrogen and helium

are the only two elements in the first row (period); this is because they only have electrons in their first shell, the 1*s* orbital. Hydrogen and helium are the only two elements that have the 1*s* and no other electron orbitals in the electrically neutral state.

The second electron shell may contain eight electrons. This shell contains another spherical *s* orbital and three "dumbbell" shaped *p* orbitals, each of which can hold two electrons, as shown in Figure **1.2.7**. After the 1*s* orbital is filled, the second electron shell is filled, first filling its 2*s* orbital and then its three *p* orbitals. When filling the *p* orbitals, each takes a single electron; once each *p* orbital has an electron, a second may be added. Lithium (Li) contains three electrons that occupy the first and second shells. Two electrons fill the 1*s* orbital, and the third electron then fills the 2*s* orbital. Its electron configuration is $1s^2 2s^1$. Neon (Ne), on the other hand, has a total of ten electrons: two are in its innermost 1*s* orbital and eight fill its second shell (two each in the 2*s* and three *p* orbitals); thus, it is an inert gas and energetically stable as a single atom that will rarely form a chemical bond with other atoms. Larger elements have additional orbitals, making up the third electron shell. While the concepts of electron shells and orbitals are closely related, orbitals provide a more accurate depiction of the electron configuration of an atom because the orbital model specifies the different shapes and special orientations of all the places that electrons may occupy.

Link to Learning



Video: Watch this visual animation to see the spatial arrangement of the p and s orbitals.

Chemical Reactions and Molecules

All elements are most stable when their outermost shell is filled with electrons according to the octet rule. This is because it is energetically favorable for atoms to be in that configuration and it makes them stable. However, since not all elements have enough electrons to fill their outermost shells, atoms form chemical bonds with other atoms thereby obtaining the electrons they need to attain a stable electron configuration. When two or more atoms chemically bond with each other, the resultant chemical structure is a molecule. The familiar water molecule, H_2O, consists of two hydrogen atoms and one oxygen atom; these bond together to form water, as illustrated in Figure **1.2.8**. Atoms can form molecules by donating, accepting, or sharing electrons to fill their outer shells.

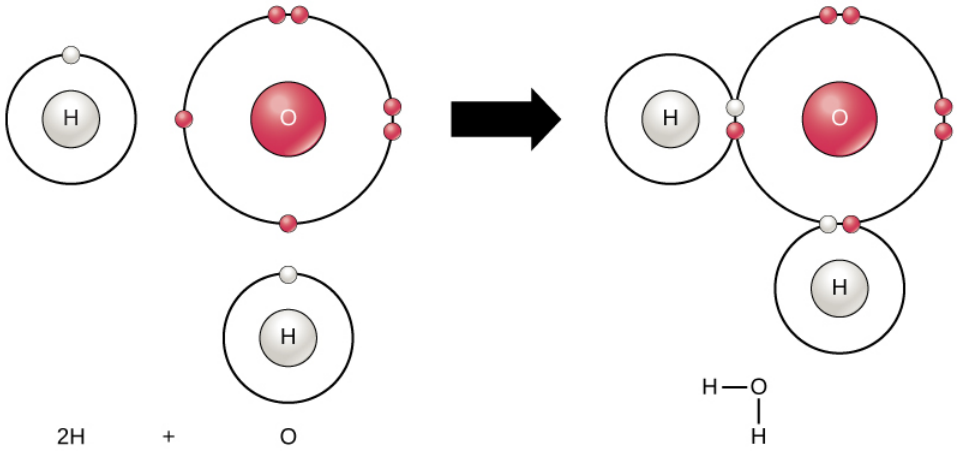


Figure **1.2.8**: Two or more atoms may bond with each other to form a molecule. When two hydrogens and an oxygen share electrons via covalent bonds, a water molecule is formed.

Chemical reactions occur when two or more atoms bond together to form molecules or when bonded atoms are broken apart. The substances used in the beginning of a chemical reaction are called the reactants (usually found on the left side of a chemical equation), and the substances found at the end of the reaction are known as the products (usually found on the right side of a chemical equation). An arrow is typically drawn between the reactants and products to indicate the direction of the chemical reaction; this direction is not always a "one-way street." For the creation of the water molecule shown above, the chemical equation would be:

$$2\,H + O \rightarrow H_2O$$

An example of a simple chemical reaction is the breaking down of hydrogen peroxide molecules, each of which consists of two hydrogen atoms bonded to two oxygen atoms (H_2O_2). The reactant hydrogen peroxide is broken down into water, containing one oxygen atom bound to two hydrogen atoms (H_2O), and oxygen, which consists of two bonded oxygen atoms (O_2). In the equation below, the reaction includes two hydrogen peroxide molecules and two water molecules. This is an example of a balanced chemical equation, wherein the number of atoms of each element is the same on each side of the equation. According to the law of conservation of matter, the number of atoms before and after a chemical reaction should be equal, such that no atoms are, under normal circumstances, created or destroyed.

$$2\,H_2O_2\ (\text{hydrogen peroxide}) \rightarrow 2\,H_2O\ (\text{water}) + O_2\ (\text{oxygen})$$

Even though all of the reactants and products of this reaction are molecules (each atom remains bonded to at least one other atom), in this reaction only hydrogen peroxide and water are representatives of compounds: they contain atoms of more than one type of element. Molecular oxygen, on the other hand, as shown in Figure **1.2.9**, consists of two doubly bonded oxygen atoms and is not classified as a compound but as a mononuclear molecule.

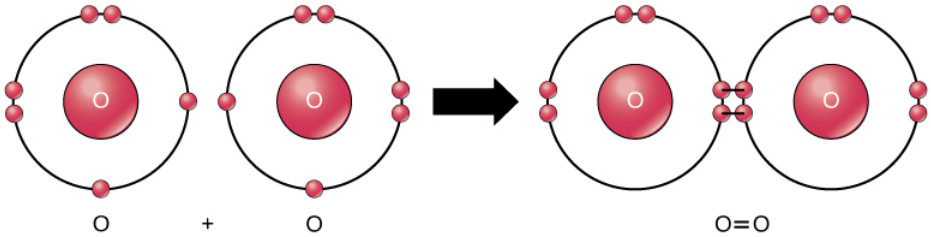


Figure **1.2.9**: The oxygen atoms in an O_2 molecule are joined by a double bond.

Some chemical reactions, such as the one shown above, can proceed in one direction until the reactants are all used up. The equations that describe these reactions contain a unidirectional arrow and are irreversible. Reversible reactions are those that can go in either direction. In reversible reactions, reactants are turned into products, but when the concentration of product goes beyond a certain threshold (characteristic of the particular reaction), some of these products will be converted back into reactants; at this point, the designations of products and reactants are reversed. This back and forth continues until a certain relative balance between

reactants and products occurs—a state called equilibrium. These situations of reversible reactions are often denoted by a chemical equation with a double headed arrow pointing towards both the reactants and products.

For example, in human blood, excess hydrogen ions (H^+) bind to bicarbonate ions (HCO_3^-) forming an equilibrium state with carbonic acid (H_2CO_3). If carbonic acid were added to this system, some of it would be converted to bicarbonate and hydrogen ions.

$$HCO_3^- + H^+ \leftrightarrow H_2CO_3$$

In biological reactions, however, equilibrium is rarely obtained because the concentrations of the reactants or products or both are constantly changing, often with a product of one reaction being a reactant for another. To return to the example of excess hydrogen ions in the blood, the formation of carbonic acid will be the major direction of the reaction. However, the carbonic acid can also leave the body as carbon dioxide gas (via exhalation) instead of being converted back to bicarbonate ion, thus driving the reaction to the right by the chemical law known as law of mass action. These reactions are important for maintaining the homeostasis of our blood.

$$HCO_3^- + H^+ \leftrightarrow H_2CO_3 \leftrightarrow CO_2 + H_2O$$

Ions and Ionic Bonds

Some atoms are more stable when they gain or lose an electron (or possibly two) and form ions. This fills their outermost electron shell and makes them energetically more stable. Because the number of electrons does not equal the number of protons, each ion has a net charge. Cations are positive ions that are formed by losing electrons. Negative ions are formed by gaining electrons and are called anions. Anions are designated by their elemental name being altered to end in "-ide": the anion of chlorine is called chloride, and the anion of sulfur is called sulfide, for example.

This movement of electrons from one element to another is referred to as electron transfer. As Figure **1.2.10** illustrates, sodium (Na) only has one electron in its outer electron shell. It takes less energy for sodium to donate that one electron than it does to accept seven more electrons to fill the outer shell. If sodium loses an electron, it now has 11 protons, 11 neutrons, and only 10 electrons, leaving it with an overall charge of +1. It is now referred to as a sodium ion. Chlorine (Cl) in its lowest energy state (called the ground state) has seven electrons in its outer shell. Again, it is more energy-efficient for chlorine to gain one electron than to lose seven. Therefore, it tends to gain an electron to create an ion with 17 protons, 17 neutrons, and 18 electrons, giving it a net negative (–1) charge. It is now referred to as a chloride ion. In this example, sodium will donate its one electron to empty its shell, and chlorine will accept that electron to fill its shell. Both ions now satisfy the octet rule and have complete outermost shells. Because the number of electrons is no longer equal to the number of protons, each is now an ion and has a +1 (sodium cation) or –1 (chloride anion) charge. Note that these transactions can normally only take place simultaneously: in order for a sodium atom to lose an electron, it must be in the presence of a suitable recipient like a chlorine atom.

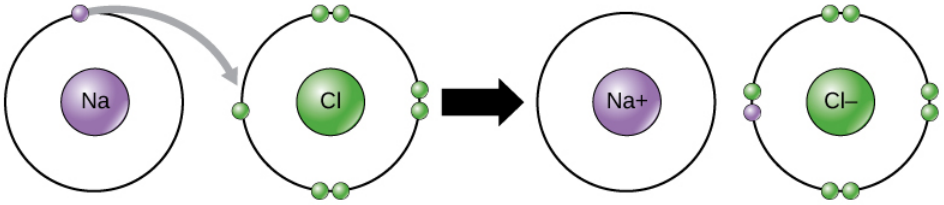


Figure **1.2.10**: In the formation of an ionic compound, metals lose electrons and nonmetals gain electrons to achieve an octet.

Ionic bonds are formed between ions with opposite charges. For instance, positively charged sodium ions and negatively charged chloride ions bond together to make crystals of sodium chloride, or table salt, creating a crystalline molecule with zero net charge.

Certain salts are referred to in physiology as electrolytes (including sodium, potassium, and calcium), ions necessary for nerve impulse conduction, muscle contractions and water balance. Many sports drinks and dietary supplements provide these ions to replace those lost from the body via sweating during exercise.

Covalent Bonds and Other Bonds and Interactions

Another way the octet rule can be satisfied is by the sharing of electrons between atoms to form covalent bonds. These bonds are stronger and much more common than ionic bonds in the molecules of living organisms. Covalent bonds are commonly found in

carbon-based organic molecules, such as our DNA and proteins. Covalent bonds are also found in inorganic molecules like H_2O, CO_2, and O_2. One, two, or three pairs of electrons may be shared, making single, double, and triple bonds, respectively. The more covalent bonds between two atoms, the stronger their connection. Thus, triple bonds are the strongest.

The strength of different levels of covalent bonding is one of the main reasons living organisms have a difficult time in acquiring nitrogen for use in constructing their molecules, even though molecular nitrogen, N_2, is the most abundant gas in the atmosphere. Molecular nitrogen consists of two nitrogen atoms triple bonded to each other and, as with all molecules, the sharing of these three pairs of electrons between the two nitrogen atoms allows for the filling of their outer electron shells, making the molecule more stable than the individual nitrogen atoms. This strong triple bond makes it difficult for living systems to break apart this nitrogen in order to use it as constituents of proteins and DNA.

The formation of water molecules provides an example of covalent bonding. The hydrogen and oxygen atoms that combine to form water molecules are bound together by covalent bonds, as shown in Figure **1.2.8**. The electron from the hydrogen splits its time between the incomplete outer shell of the hydrogen atoms and the incomplete outer shell of the oxygen atoms. To completely fill the outer shell of oxygen, which has six electrons in its outer shell but which would be more stable with eight, two electrons (one from each hydrogen atom) are needed: hence the well-known formula H_2O. The electrons are shared between the two elements to fill the outer shell of each, making both elements more stable.

Link to Learning



Video: View this short video to see an animation of ionic and covalent bonding.

Polar Covalent Bonds

There are two types of covalent bonds: polar and nonpolar. In a polar covalent bond, shown in Figure **1.2.11**, the electrons are unequally shared by the atoms and are attracted more to one nucleus than the other. Because of the unequal distribution of electrons between the atoms of different elements, a slightly positive (δ+) or slightly negative (δ–) charge develops. This partial charge is an important property of water and accounts for many of its characteristics.

Water is a polar molecule, with the hydrogen atoms acquiring a partial positive charge and the oxygen a partial negative charge. This occurs because the nucleus of the oxygen atom is more attractive to the electrons of the hydrogen atoms than the hydrogen nucleus is to the oxygen's electrons. Thus oxygen has a higher electronegativity than hydrogen and the shared electrons spend more time in the vicinity of the oxygen nucleus than they do near the nucleus of the hydrogen atoms, giving the atoms of oxygen and hydrogen slightly negative and positive charges, respectively. Another way of stating this is that the probability of finding a shared electron near an oxygen nucleus is more likely than finding it near a hydrogen nucleus. Either way, the atom's relative electronegativity contributes to the development of partial charges whenever one element is significantly more electronegative than the other, and the charges generated by these polar bonds may then be used for the formation of hydrogen bonds based on the

attraction of opposite partial charges. (Hydrogen bonds, which are discussed in detail below, are weak bonds between slightly positively charged hydrogen atoms to slightly negatively charged atoms in other molecules.) Since macromolecules often have atoms within them that differ in electronegativity, polar bonds are often present in organic molecules.

Nonpolar Covalent Bonds

Nonpolar covalent bonds form between two atoms of the same element or between different elements that share electrons equally. For example, molecular oxygen (O_2) is nonpolar because the electrons will be equally distributed between the two oxygen atoms.

Another example of a nonpolar covalent bond is methane (CH_4), also shown in Figure **1.2.11**. Carbon has four electrons in its outermost shell and needs four more to fill it. It gets these four from four hydrogen atoms, each atom providing one, making a stable outer shell of eight electrons. Carbon and hydrogen do not have the same electronegativity but are similar; thus, nonpolar bonds form. The hydrogen atoms each need one electron for their outermost shell, which is filled when it contains two electrons. These elements share the electrons equally among the carbons and the hydrogen atoms, creating a nonpolar covalent molecule.

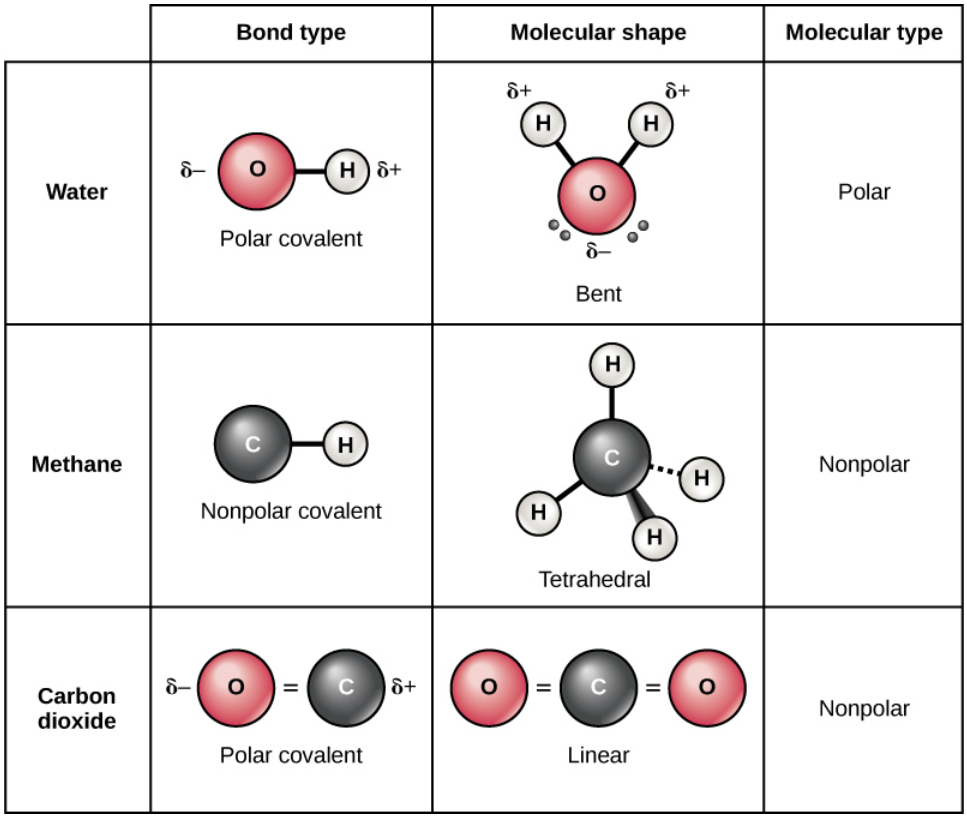


Figure **1.2.11**: Whether a molecule is polar or nonpolar depends both on bond type and molecular shape. Both water and carbon dioxide have polar covalent bonds, but carbon dioxide is linear, so the partial charges on the molecule cancel each other out.

Hydrogen Bonds and Van Der Waals Interactions

Ionic and covalent bonds between elements require energy to break. Ionic bonds are not as strong as covalent, which determines their behavior in biological systems. However, not all bonds are ionic or covalent bonds. Weaker bonds can also form between molecules. Two weak bonds that occur frequently are hydrogen bonds and van der Waals interactions. Without these two types of bonds, life as we know it would not exist. Hydrogen bonds provide many of the critical, life-sustaining properties of water and also stabilize the structures of proteins and DNA, the building block of cells.

When polar covalent bonds containing hydrogen form, the hydrogen in that bond has a slightly positive charge because hydrogen's electron is pulled more strongly toward the other element and away from the hydrogen. Because the hydrogen is slightly positive, it will be attracted to neighboring negative charges. When this happens, a weak interaction occurs between the $\delta^+$of the hydrogen from one molecule and the δ– charge on the more electronegative atoms of another molecule, usually oxygen or nitrogen, or within the same molecule. This interaction is called a hydrogen bond. This type of bond is common and occurs regularly between water

molecules. Individual hydrogen bonds are weak and easily broken; however, they occur in very large numbers in water and in organic polymers, creating a major force in combination. Hydrogen bonds are also responsible for zipping together the DNA double helix.

Like hydrogen bonds, van der Waals interactions are weak attractions or interactions between molecules. Van der Waals attractions can occur between any two or more molecules and are dependent on slight fluctuations of the electron densities, which are not always symmetrical around an atom. For these attractions to happen, the molecules need to be very close to one another. These bonds—along with ionic, covalent, and hydrogen bonds—contribute to the three-dimensional structure of the proteins in our cells that is necessary for their proper function.

Career Connection: Pharmaceutical Chemist

Pharmaceutical chemists are responsible for the development of new drugs and trying to determine the mode of action of both old and new drugs. They are involved in every step of the drug development process. Drugs can be found in the natural environment or can be synthesized in the laboratory. In many cases, potential drugs found in nature are changed chemically in the laboratory to make them safer and more effective, and sometimes synthetic versions of drugs substitute for the version found in nature.

After the initial discovery or synthesis of a drug, the chemist then develops the drug, perhaps chemically altering it, testing it to see if the drug is toxic, and then designing methods for efficient large-scale production. Then, the process of getting the drug approved for human use begins. In the United States, drug approval is handled by the Food and Drug Administration (FDA) and involves a series of large-scale experiments using human subjects to make sure the drug is not harmful and effectively treats the condition it aims to treat. This process often takes several years and requires the participation of physicians and scientists, in addition to chemists, to complete testing and gain approval.

An example of a drug that was originally discovered in a living organism is Paclitaxel (Taxol), an anti-cancer drug used to treat breast cancer. This drug was discovered in the bark of the pacific yew tree. Another example is aspirin, originally isolated from willow tree bark. Finding drugs often means testing hundreds of samples of plants, fungi, and other forms of life to see if any biologically active compounds are found within them. Sometimes, traditional medicine can give modern medicine clues to where an active compound can be found. For example, the use of willow bark to make medicine has been known for thousands of years, dating back to ancient Egypt. It was not until the late 1800s, however, that the aspirin molecule, known as acetylsalicylic acid, was purified and marketed for human use.

Occasionally, drugs developed for one use are found to have unforeseen effects that allow these drugs to be used in other, unrelated ways. For example, the drug minoxidil (Rogaine) was originally developed to treat high blood pressure. When tested on humans, it was noticed that individuals taking the drug would grow new hair. Eventually the drug was marketed to men and women with baldness to restore lost hair.

The career of the pharmaceutical chemist may involve detective work, experimentation, and drug development, all with the goal of making human beings healthier.

Summary

Matter is anything that occupies space and has mass. It is made up of elements. All of the 92 elements that occur naturally have unique qualities that allow them to combine in various ways to create molecules, which in turn combine to form cells, tissues, organ systems, and organisms. Atoms, which consist of protons, neutrons, and electrons, are the smallest units of an element that retain all of the properties of that element. Electrons can be transferred, shared, or cause charge disparities between atoms to create bonds, including ionic, covalent, and hydrogen bonds, as well as van der Waals interactions.

Art Connections

[link] How many neutrons do carbon-12 and carbon-13 have, respectively?

[link] Carbon-12 has six neutrons. Carbon-13 has seven neutrons.

[link] An atom may give, take, or share electrons with another atom to achieve a full valence shell, the most stable electron configuration. Looking at this figure, how many electrons do elements in group 1 need to lose in order to achieve a stable electron configuration? How many electrons do elements in groups 14 and 17 need to gain to achieve a stable configuration?

[link] Elements in group 1 need to lose one electron to achieve a stable electron configuration. Elements in groups 14 and 17 need to gain four and one electrons, respectively, to achieve a stable configuration.

Review Questions

If xenon has an atomic number of 54 and a mass number of 108, how many neutrons does it have?

A. 54
B. 27
C. 100
D. 108

A

Atoms that vary in the number of neutrons found in their nuclei are called _____.

A. ions
B. neutrons
C. neutral atoms
D. isotopes

D

Potassium has an atomic number of 19. What is its electron configuration?

A. shells 1 and 2 are full, and shell 3 has nine electrons
B. shells 1, 2 and 3 are full and shell 4 has three electrons
C. shells 1, 2 and 3 are full and shell 4 has one electron
D. shells 1, 2 and 3 are full and no other electrons are present

C

Which type of bond represents a weak chemical bond?

A. hydrogen bond
B. atomic bond
C. covalent bond
D. nonpolar covalent bond

A

Free Response

What makes ionic bonds different from covalent bonds?

Ionic bonds are created between ions. The electrons are not shared between the atoms, but rather are associated more with one ion than the other. Ionic bonds are strong bonds, but are weaker than covalent bonds, meaning it takes less energy to break an ionic bond compared with a covalent one.

Why are hydrogen bonds and van der Waals interactions necessary for cells?

Hydrogen bonds and van der Waals interactions form weak associations between different molecules or within different regions of the same molecule. They provide the structure and shape necessary for proteins and DNA within cells so that they function properly.

Glossary

anion
negative ion that is formed by an atom gaining one or more electrons

atom
the smallest unit of matter that retains all of the chemical properties of an element

atomic mass
calculated mean of the mass number for an element's isotopes

atomic number
total number of protons in an atom

balanced chemical equation
statement of a chemical reaction with the number of each type of atom equalized for both the products and reactants

cation
positive ion that is formed by an atom losing one or more electrons

chemical bond
interaction between two or more of the same or different atoms that results in the formation of molecules

chemical reaction
process leading to the rearrangement of atoms in molecules

chemical reactivity
the ability to combine and to chemically bond with each other

compound
substance composed of molecules consisting of atoms of at least two different elements

covalent bond
type of strong bond formed between two of the same or different elements; forms when electrons are shared between atoms

electrolyte
ion necessary for nerve impulse conduction, muscle contractions and water balance

electron
negatively charged subatomic particle that resides outside of the nucleus in the electron orbital; lacks functional mass and has a negative charge of –1 unit

electron configuration
arrangement of electrons in an atom's electron shell (for example, $1s^2 2s^2 2p^6$)

electron orbital
how electrons are spatially distributed surrounding the nucleus; the area where an electron is most likely to be found

electron transfer
movement of electrons from one element to another; important in creation of ionic bonds

electronegativity
ability of some elements to attract electrons (often of hydrogen atoms), acquiring partial negative charges in molecules and creating partial positive charges on the hydrogen atoms

element
one of 118 unique substances that cannot be broken down into smaller substances; each element has unique properties and a specified number of protons

equilibrium
steady state of relative reactant and product concentration in reversible chemical reactions in a closed system

hydrogen bond
weak bond between slightly positively charged hydrogen atoms to slightly negatively charged atoms in other molecules

inert gas
(also, noble gas) element with filled outer electron shell that is unreactive with other atoms

ion
atom or chemical group that does not contain equal numbers of protons and electrons

ionic bond
chemical bond that forms between ions with opposite charges (cations and anions)

irreversible chemical reaction
chemical reaction where reactants proceed uni-directionally to form products

isotope
one or more forms of an element that have different numbers of neutrons

law of mass action
chemical law stating that the rate of a reaction is proportional to the concentration of the reacting substances

mass number
total number of protons and neutrons in an atom

matter
anything that has mass and occupies space

molecule
two or more atoms chemically bonded together

neutron
uncharged particle that resides in the nucleus of an atom; has a mass of one amu

noble gas
see inert gas

nonpolar covalent bond
type of covalent bond that forms between atoms when electrons are shared equally between them

nucleus
core of an atom; contains protons and neutrons

octet rule

rule that atoms are most stable when they hold eight electrons in their outermost shells

orbital
region surrounding the nucleus; contains electrons

periodic table
organizational chart of elements indicating the atomic number and atomic mass of each element; provides key information about the properties of the elements

polar covalent bond
type of covalent bond that forms as a result of unequal sharing of electrons, resulting in the creation of slightly positive and slightly negative charged regions of the molecule

product
molecule found on the right side of a chemical equation

proton
positively charged particle that resides in the nucleus of an atom; has a mass of one amu and a charge of +1

radioisotope
isotope that emits radiation composed of subatomic particles to form more stable elements

reactant
molecule found on the left side of a chemical equation

reversible chemical reaction
chemical reaction that functions bi-directionally, where products may turn into reactants if their concentration is great enough

valence shell
outermost shell of an atom

van der Waals interaction
very weak interaction between molecules due to temporary charges attracting atoms that are very close together

Contributors and Attributions

- Template:ContribOpenSTAXBio

This page titled 1.2: Atoms, Ions, Molecules is shared under a CC BY license and was authored, remixed, and/or curated by OpenStax.

- **2.1: Atoms, Isotopes, Ions, and Molecules - The Building Blocks** by OpenStax is licensed CC BY 4.0.

1.3: Intermolecular Forces

Learning Objectives

- Describe the types of intermolecular forces possible between atoms or molecules in condensed phases (dispersion forces, dipole-dipole attractions, and hydrogen bonding)
- Identify the types of intermolecular forces experienced by specific molecules based on their structures
- Explain the relation between the intermolecular forces present within a substance and the temperatures associated with changes in its physical state

As was the case for gaseous substances, the kinetic molecular theory may be used to explain the behavior of solids and liquids. In the following description, the term *particle* will be used to refer to an atom, molecule, or ion. Note that we will use the popular phrase "intermolecular attraction" to refer to attractive forces between the particles of a substance, regardless of whether these particles are molecules, atoms, or ions.

Consider these two aspects of the molecular-level environments in solid, liquid, and gaseous matter:

- Particles in a solid are tightly packed together and often arranged in a regular pattern; in a liquid, they are close together with no regular arrangement; in a gas, they are far apart with no regular arrangement.
- Particles in a solid vibrate about fixed positions and do not generally move in relation to one another; in a liquid, they move past each other but remain in essentially constant contact; in a gas, they move independently of one another except when they collide.

The differences in the properties of a solid, liquid, or gas reflect the strengths of the attractive forces between the atoms, molecules, or ions that make up each phase. The phase in which a substance exists depends on the relative extents of its intermolecular forces (IMFs) and the kinetic energies (KE) of its molecules. IMFs are the various forces of attraction that may exist between the atoms and molecules of a substance due to electrostatic phenomena, as will be detailed in this module. These forces serve to hold particles close together, whereas the particles' KE provides the energy required to overcome the attractive forces and thus increase the distance between particles. Figure 1.3.1 illustrates how changes in physical state may be induced by changing the temperature, hence, the average KE, of a given substance.

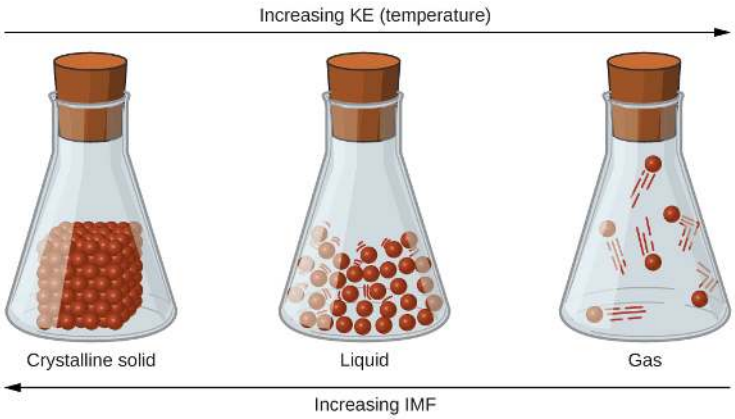


Figure 1.3.1: Transitions between solid, liquid, and gaseous states of a substance occur when conditions of temperature or pressure favor the associated changes in intermolecular forces. (Note: The space between particles in the gas phase is much greater than shown.)

As an example of the processes depicted in this figure, consider a sample of water. When gaseous water is cooled sufficiently, the attractions between H_2O molecules will be capable of holding them together when they come into contact with each other; the gas condenses, forming liquid H_2O. For example, liquid water forms on the outside of a cold glass as the water vapor in the air is cooled by the cold glass, as seen in Figure 1.3.2.



Figure **1.3.2**: Condensation forms when water vapor in the air is cooled enough to form liquid water, such as (a) on the outside of a cold beverage glass or (b) in the form of fog. (credit a: modification of work by Jenny Downing; credit b: modification of work by Cory Zanker)

We can also liquefy many gases by compressing them, if the temperature is not too high. The increased pressure brings the molecules of a gas closer together, such that the attractions between the molecules become strong relative to their KE. Consequently, they form liquids. Butane, C_4H_{10}, is the fuel used in disposable lighters and is a gas at standard temperature and pressure. Inside the lighter's fuel compartment, the butane is compressed to a pressure that results in its condensation to the liquid state, as shown in Figure **1.3.3**.



Figure **1.3.3**: Gaseous butane is compressed within the storage compartment of a disposable lighter, resulting in its condensation to the liquid state. (credit: modification of work by "Sam-Cat"/Flickr)

Finally, if the temperature of a liquid becomes sufficiently low, or the pressure on the liquid becomes sufficiently high, the molecules of the liquid no longer have enough KE to overcome the IMF between them, and a solid forms. A more thorough discussion of these and other changes of state, or phase transitions, is provided in a later module of this chapter.

Forces between Molecules

Under appropriate conditions, the attractions between all gas molecules will cause them to form liquids or solids. This is due to intermolecular forces, not *intra*molecular forces. *Intra*molecular forces are those *within* the molecule that keep the molecule together, for example, the bonds between the atoms. *Inter*molecular forces are the attractions *between* molecules, which determine many of the physical properties of a substance. Figure **1.3.4** illustrates these different molecular forces. The strengths of these attractive forces vary widely, though usually the IMFs between small molecules are weak compared to the intramolecular forces that bond atoms together within a molecule. For example, to overcome the IMFs in one mole of liquid HCl and convert it into

gaseous HCl requires only about 17 kilojoules. However, to break the covalent bonds between the hydrogen and chlorine atoms in one mole of HCl requires about 25 times more energy—430 kilojoules.

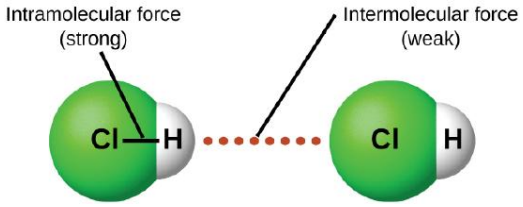


Figure **1.3.4**: Intramolecular forces keep a molecule intact. Intermolecular forces hold multiple molecules together and determine many of a substance's properties.

All of the attractive forces between neutral atoms and molecules are known as van der Waals forces, although they are usually referred to more informally as intermolecular attraction. We will consider the various types of IMFs in the next three sections of this module.

Dispersion Forces

One of the three van der Waals forces is present in all condensed phases, regardless of the nature of the atoms or molecules composing the substance. This attractive force is called the London dispersion force in honor of German-born American physicist Fritz London who, in 1928, first explained it. This force is often referred to as simply the dispersion force. Because the electrons of an atom or molecule are in constant motion (or, alternatively, the electron's location is subject to quantum-mechanical variability), at any moment in time, an atom or molecule can develop a temporary, instantaneous dipole if its electrons are distributed asymmetrically. The presence of this dipole can, in turn, distort the electrons of a neighboring atom or molecule, producing an induced dipole. These two rapidly fluctuating, temporary dipoles thus result in a relatively weak electrostatic attraction between the species—a so-called dispersion force like that illustrated in Figure **1.3.5**.

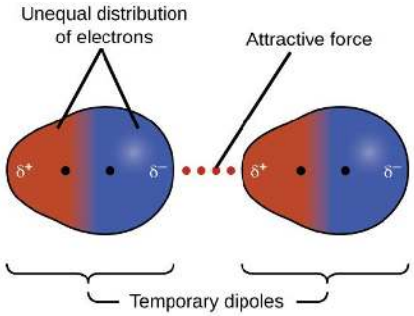


Figure **1.3.5**: Dispersion forces result from the formation of temporary dipoles, as illustrated here for two nonpolar diatomic molecules.

Dispersion forces that develop between atoms in different molecules can attract the two molecules to each other. The forces are relatively weak, however, and become significant only when the molecules are very close. Larger and heavier atoms and molecules exhibit stronger dispersion forces than do smaller and lighter atoms and molecules. F_2 and Cl_2 are gases at room temperature (reflecting weaker attractive forces); Br_2 is a liquid, and I_2 is a solid (reflecting stronger attractive forces). Trends in observed melting and boiling points for the halogens clearly demonstrate this effect, as seen in Table **1.3.1**.

Table **1.3.1**: Melting and Boiling Points of the Halogens

Halogen	Molar Mass	Atomic Radius	Melting Point	Boiling Point
fluorine, F_2	38 g/mol	72 pm	53 K	85 K
chlorine, Cl_2	71 g/mol	99 pm	172 K	238 K
bromine, Br_2	160 g/mol	114 pm	266 K	332 K
iodine, I_2	254 g/mol	133 pm	387 K	457 K
astatine, At_2	420 g/mol	150 pm	575 K	610 K

The increase in melting and boiling points with increasing atomic/molecular size may be rationalized by considering how the strength of dispersion forces is affected by the electronic structure of the atoms or molecules in the substance. In a larger atom, the valence electrons are, on average, farther from the nuclei than in a smaller atom. Thus, they are less tightly held and can more easily form the temporary dipoles that produce the attraction. The measure of how easy or difficult it is for another electrostatic charge (for example, a nearby ion or polar molecule) to distort a molecule's charge distribution (its electron cloud) is known as polarizability. A molecule that has a charge cloud that is easily distorted is said to be very polarizable and will have large dispersion forces; one with a charge cloud that is difficult to distort is not very polarizable and will have small dispersion forces.

Example 1.3.1: London Forces and Their Effects

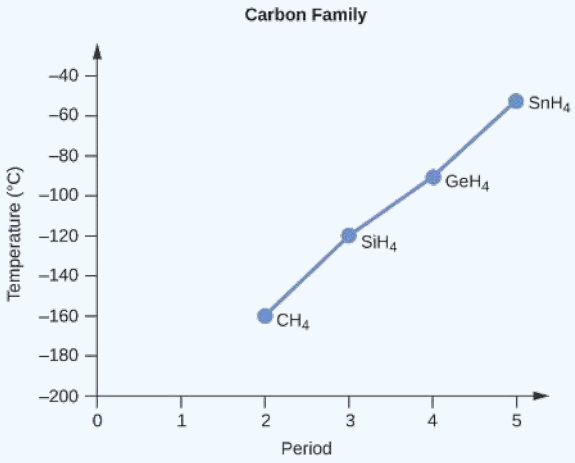
Order the following compounds of a group 14 element and hydrogen from lowest to highest boiling point: CH_4, SiH_4, GeH_4, and SnH_4. Explain your reasoning.

Solution

Applying the skills acquired in the chapter on chemical bonding and molecular geometry, all of these compounds are predicted to be nonpolar, so they may experience only dispersion forces: the smaller the molecule, the less polarizable and the weaker the dispersion forces; the larger the molecule, the larger the dispersion forces. The molar masses of CH_4, SiH_4, GeH_4, and SnH_4 are approximately 16 g/mol, 32 g/mol, 77 g/mol, and 123 g/mol, respectively. Therefore, CH_4 is expected to have the lowest boiling point and SnH_4 the highest boiling point. The ordering from lowest to highest boiling point is expected to be

$$CH_4 < SiH_4 < GeH_4 < SnH_4$$

A graph of the actual boiling points of these compounds versus the period of the group 14 elements shows this prediction to be correct:



Exercise 1.3.1

Order the following hydrocarbons from lowest to highest boiling point: C_2H_6, C_3H_8, and C_4H_{10}.

Answer

All of these compounds are nonpolar and only have London dispersion forces: the larger the molecule, the larger the dispersion forces and the higher the boiling point. The ordering from lowest to highest boiling point is therefore

$$C_2H_6 < C_3H_8 < C_4H_{10}.$$

The shapes of molecules also affect the magnitudes of the dispersion forces between them. For example, boiling points for the isomers *n*-pentane, isopentane, and neopentane (shown in Figure **1.3.6**) are 36 °C, 27 °C, and 9.5 °C, respectively. Even though these compounds are composed of molecules with the same chemical formula, C_5H_{12}, the difference in boiling points suggests that dispersion forces in the liquid phase are different, being greatest for *n*-pentane and least for neopentane. The elongated shape of *n*-

pentane provides a greater surface area available for contact between molecules, resulting in correspondingly stronger dispersion forces. The more compact shape of isopentane offers a smaller surface area available for intermolecular contact and, therefore, weaker dispersion forces. Neopentane molecules are the most compact of the three, offering the least available surface area for intermolecular contact and, hence, the weakest dispersion forces. This behavior is analogous to the connections that may be formed between strips of VELCRO brand fasteners: the greater the area of the strip's contact, the stronger the connection.

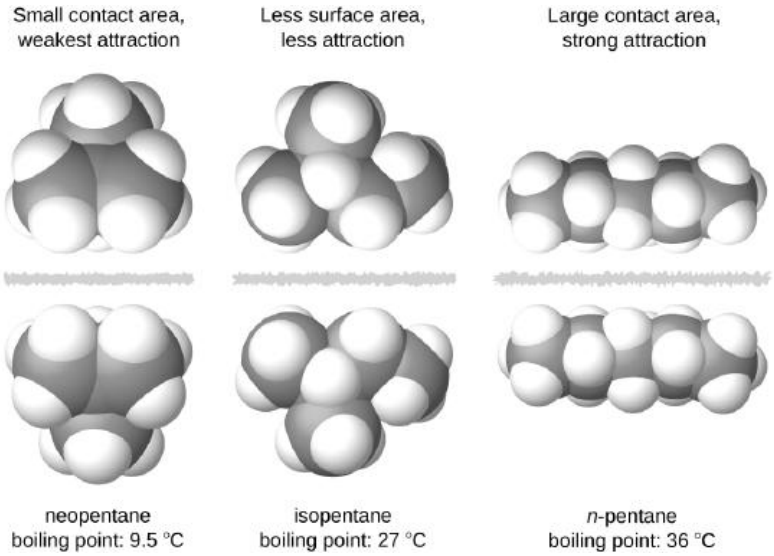


Figure **1.3.6**: The strength of the dispersion forces increases with the contact area between molecules, as demonstrated by the boiling points of these pentane isomers.

Applications: Geckos and Intermolecular Forces

Geckos have an amazing ability to adhere to most surfaces. They can quickly run up smooth walls and across ceilings that have no toe-holds, and they do this without having suction cups or a sticky substance on their toes. And while a gecko can lift its feet easily as it walks along a surface, if you attempt to pick it up, it sticks to the surface. How are geckos (as well as spiders and some other insects) able to do this? Although this phenomenon has been investigated for hundreds of years, scientists only recently uncovered the details of the process that allows geckos' feet to behave this way.

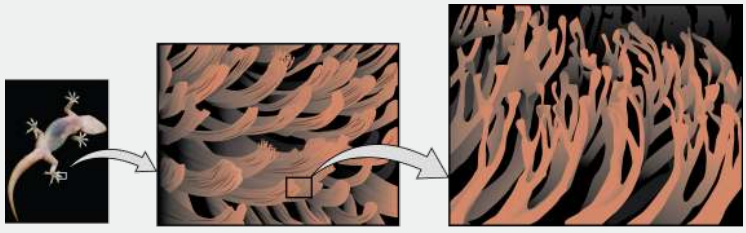


Figure **1.3.7**: Geckos' toes contain large numbers of tiny hairs (setae), which branch into many triangular tips (spatulae). Geckos adhere to surfaces because of van der Waals attractions between the surface and a gecko's millions of spatulae. By changing how the spatulae contact the surface, geckos can turn their stickiness "on" and "off." (credit photo: modification of work by "JC*+A!"/Flickr)

Geckos' toes are covered with hundreds of thousands of tiny hairs known as *setae*, with each seta, in turn, branching into hundreds of tiny, flat, triangular tips called *spatulae*. The huge numbers of spatulae on its setae provide a gecko, shown in Figure **1.3.7**, with a large total surface area for sticking to a surface. In 2000, Kellar Autumn, who leads a multi-institutional gecko research team, found that geckos adhered equally well to both polar silicon dioxide and nonpolar gallium arsenide. This proved that geckos stick to surfaces because of dispersion forces—weak intermolecular attractions arising from temporary, synchronized charge distributions between adjacent molecules. Although dispersion forces are very weak, the total attraction over millions of spatulae is large enough to support many times the gecko's weight.

In 2014, two scientists developed a model to explain how geckos can rapidly transition from “sticky” to “non-sticky.” Alex Greaney and Congcong Hu at Oregon State University described how geckos can achieve this by changing the angle between their spatulae and the surface. Geckos’ feet, which are normally nonsticky, become sticky when a small shear force is applied. By curling and uncurling their toes, geckos can alternate between sticking and unsticking from a surface, and thus easily move across it. Further investigations may eventually lead to the development of better adhesives and other applications.



Watch this video to learn more about Kellar Autumn’s research that determined that van der Waals forces are responsible for a gecko’s ability to cling and climb.

Dipole-Dipole Attractions

Recall from the chapter on chemical bonding and molecular geometry that *polar* molecules have a partial positive charge on one side and a partial negative charge on the other side of the molecule—a separation of charge called a *dipole*. Consider a polar molecule such as hydrogen chloride, HCl. In the HCl molecule, the more electronegative Cl atom bears the partial negative charge, whereas the less electronegative H atom bears the partial positive charge. An attractive force between HCl molecules results from the attraction between the positive end of one HCl molecule and the negative end of another. This attractive force is called a dipole-dipole attraction—the electrostatic force between the partially positive end of one polar molecule and the partially negative end of another, as illustrated in Figure **1.3.8**.

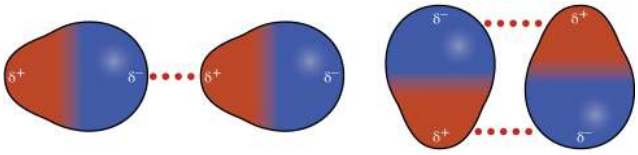


Figure **1.3.8**: This image shows two arrangements of polar molecules, such as HCl, that allow an attraction between the partial negative end of one molecule and the partial positive end of another.

The effect of a dipole-dipole attraction is apparent when we compare the properties of HCl molecules to nonpolar F_2 molecules. Both HCl and F_2 consist of the same number of atoms and have approximately the same molecular mass. At a temperature of 150 K, molecules of both substances would have the same average KE. However, the dipole-dipole attractions between HCl molecules are sufficient to cause them to “stick together” to form a liquid, whereas the relatively weaker dispersion forces between nonpolar F_2 molecules are not, and so this substance is gaseous at this temperature. The higher normal boiling point of HCl (188 K) compared to F_2 (85 K) is a reflection of the greater strength of dipole-dipole attractions between HCl molecules, compared to the attractions between nonpolar F_2 molecules. We will often use values such as boiling or freezing points, or enthalpies of vaporization or fusion, as indicators of the relative strengths of IMFs of attraction present within different substances.

✔ Example **1.3.2**: Dipole-Dipole Forces and Their Effects

Predict which will have the higher boiling point: N_2 or CO. Explain your reasoning.

Solution

CO and N_2 are both diatomic molecules with masses of about 28 amu, so they experience similar London dispersion forces. Because CO is a polar molecule, it experiences dipole-dipole attractions. Because N_2 is nonpolar, its molecules cannot exhibit dipole-dipole attractions. The dipole-dipole attractions between CO molecules are comparably stronger than the dispersion forces between nonpolar N_2 molecules, so CO is expected to have the higher boiling point.

A common method for preparing oxygen is the decomposition

? Exercise 1.3.2

Predict which will have the higher boiling point: **ICl** or **Br_2**. Explain your reasoning.

Answer

ICl. ICl and Br_2 have similar masses (~160 amu) and therefore experience similar London dispersion forces. ICl is polar and thus also exhibits dipole-dipole attractions; Br_2 is nonpolar and does not. The relatively stronger dipole-dipole attractions require more energy to overcome, so ICl will have the higher boiling point.

Hydrogen Bonding

Nitrosyl fluoride (ONF, molecular mass 49 amu) is a gas at room temperature. Water (H_2O, molecular mass 18 amu) is a liquid, even though it has a lower molecular mass. We clearly cannot attribute this difference between the two compounds to dispersion forces. Both molecules have about the same shape and ONF is the heavier and larger molecule. It is, therefore, expected to experience more significant dispersion forces. Additionally, we cannot attribute this difference in boiling points to differences in the dipole moments of the molecules. Both molecules are polar and exhibit comparable dipole moments. The large difference between the boiling points is due to a particularly strong dipole-dipole attraction that may occur when a molecule contains a hydrogen atom bonded to a fluorine, oxygen, or nitrogen atom (the three most electronegative elements). The very large difference in electronegativity between the H atom (2.1) and the atom to which it is bonded (4.0 for an F atom, 3.5 for an O atom, or 3.0 for a N atom), combined with the very small size of a H atom and the relatively small sizes of F, O, or N atoms, leads to *highly concentrated partial charges* with these atoms. Molecules with F-H, O-H, or N-H moieties are very strongly attracted to similar moieties in nearby molecules, a particularly strong type of dipole-dipole attraction called hydrogen bonding. Examples of hydrogen bonds include HF···HF, H_2O···HOH, and H_3N···HNH_2, in which the hydrogen bonds are denoted by dots. Figure **1.3.9** illustrates hydrogen bonding between water molecules.

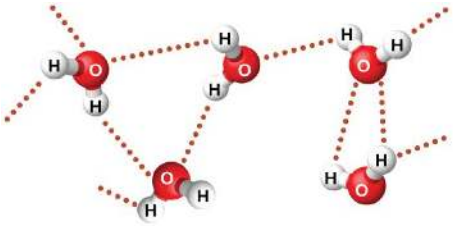


Figure **1.3.9**: Water molecules participate in multiple hydrogen-bonding interactions with nearby water molecules.

Despite use of the word “bond,” keep in mind that hydrogen bonds are *intermolecular* attractive forces, not *intramolecular* attractive forces (covalent bonds). Hydrogen bonds are much weaker than covalent bonds, only about 5 to 10% as strong, but are generally much stronger than other dipole-dipole attractions and dispersion forces.

Hydrogen bonds have a pronounced effect on the properties of condensed phases (liquids and solids). For example, consider the trends in boiling points for the binary hydrides of group 15 (NH_3, PH_3, AsH_3, and SbH_3), group 16 hydrides (H_2O, H_2S, H_2Se, and H_2Te), and group 17 hydrides (HF, HCl, HBr, and HI). The boiling points of the heaviest three hydrides for each group are plotted in Figure **1.3.10**. As we progress down any of these groups, the polarities of the molecules decrease slightly, whereas the sizes of the molecules increase substantially. The effect of increasingly stronger dispersion forces dominates that of increasingly weaker dipole-dipole attractions, and the boiling points are observed to increase steadily. For the group 15, 16, and 17 hydrides, the boiling points for each class of compounds increase with increasing molecular mass for elements in periods 3, 4, and 5.

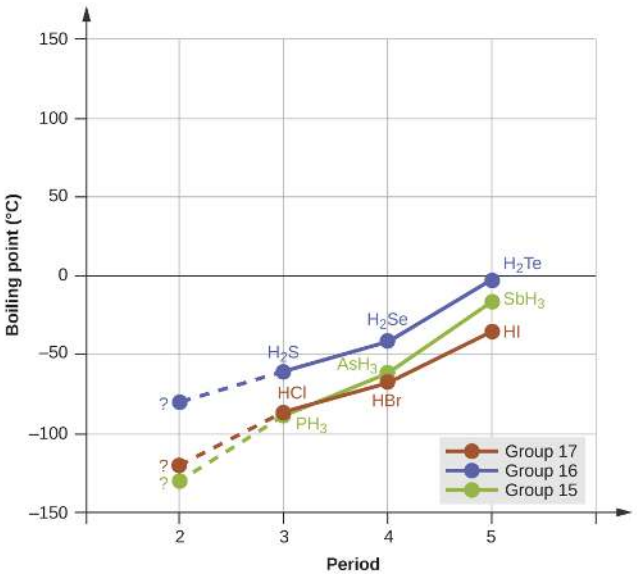


Figure 1.3.10: In comparison to periods 3−5, the binary hydrides of period 2 elements in groups 17, 16 and 15 (F, O and N, respectively) exhibit anomalously high boiling points due to hydrogen bonding.

If we use this trend to predict the boiling points for the lightest hydride for each group, we would expect NH_3 to boil at about −120 °C, H_2O to boil at about −80 °C, and HF to boil at about −110 °C. However, when we measure the boiling points for these compounds, we find that they are dramatically higher than the trends would predict, as shown in Figure 1.3.10. The stark contrast between our naïve predictions and reality provides compelling evidence for the strength of hydrogen bonding.

✔ Example 1.3.3: Effect of Hydrogen Bonding on Boiling Points

Consider the compounds dimethylether (CH_3OCH_3), ethanol (CH_3CH_2OH), and propane ($CH_3CH_2CH_3$). Their boiling points, not necessarily in order, are −42.1 °C, −24.8 °C, and 78.4 °C. Match each compound with its boiling point. Explain your reasoning.

Solution

The VSEPR-predicted shapes of CH_3OCH_3, CH_3CH_2OH, and $CH_3CH_2CH_3$ are similar, as are their molar masses (46 g/mol, 46 g/mol, and 44 g/mol, respectively), so they will exhibit similar dispersion forces. Since $CH_3CH_2CH_3$ is nonpolar, it may exhibit *only* dispersion forces. Because CH_3OCH_3 is polar, it will also experience dipole-dipole attractions. Finally, CH_3CH_2OH has an −OH group, and so it will experience the uniquely strong dipole-dipole attraction known as hydrogen bonding. So the ordering in terms of strength of IMFs, and thus boiling points, is $CH_3CH_2CH_3 < CH_3OCH_3 < CH_3CH_2OH$. The boiling point of propane is −42.1 °C, the boiling point of dimethylether is −24.8 °C, and the boiling point of ethanol is 78.5 °C.

? Exercise 1.3.2

Ethane (CH_3CH_3) has a melting point of −183 °C and a boiling point of −89 °C. Predict the melting and boiling points for methylamine (CH_3NH_2). Explain your reasoning.

Answer

The melting point and boiling point for methylamine are predicted to be significantly greater than those of ethane. CH_3CH_3 and CH_3NH_2 are similar in size and mass, but methylamine possesses an −NH group and therefore may exhibit hydrogen bonding. This greatly increases its IMFs, and therefore its melting and boiling points. It is difficult to predict values, but the known values are a melting point of −93 °C and a boiling point of −6 °C.

Hydrogen Bonding and DNA

Deoxyribonucleic acid (DNA) is found in every living organism and contains the genetic information that determines the organism's characteristics, provides the blueprint for making the proteins necessary for life, and serves as a template to pass this information on to the organism's offspring. A DNA molecule consists of two (anti-)parallel chains of repeating nucleotides, which form its well-known double helical structure, as shown in Figure 1.3.10.

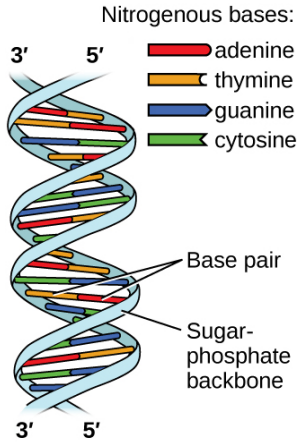


Figure 1.3.11: Two separate DNA molecules form a double-stranded helix in which the molecules are held together via hydrogen bonding. (credit: modification of work by Jerome Walker, Dennis Myts)

Each nucleotide contains a (deoxyribose) sugar bound to a phosphate group on one side, and one of four nitrogenous bases on the other. Two of the bases, cytosine (C) and thymine (T), are single-ringed structures known as pyrimidines. The other two, adenine (A) and guanine (G), are double-ringed structures called purines. These bases form complementary base pairs consisting of one purine and one pyrimidine, with adenine pairing with thymine, and cytosine with guanine. Each base pair is held together by hydrogen bonding. A and T share two hydrogen bonds, C and G share three, and both pairings have a similar shape and structure Figure 1.3.12

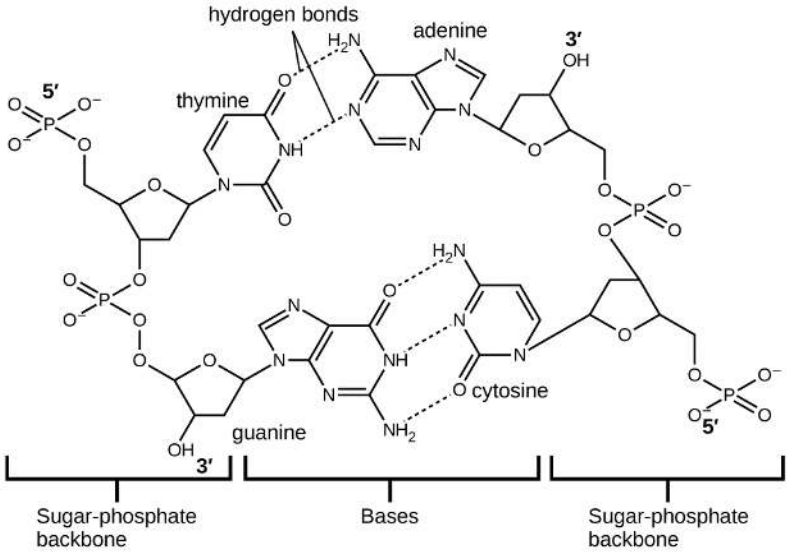


Figure 1.3.12: The geometries of the base molecules result in maximum hydrogen bonding between adenine and thymine (AT) and between guanine and cytosine (GC), so-called "complementary base pairs."

The cumulative effect of millions of hydrogen bonds effectively holds the two strands of DNA together. Importantly, the two strands of DNA can relatively easily "unzip" down the middle since hydrogen bonds are relatively weak compared to the covalent bonds that hold the atoms of the individual DNA molecules together. This allows both strands to function as a template for replication.

Summary

The physical properties of condensed matter (liquids and solids) can be explained in terms of the kinetic molecular theory. In a liquid, intermolecular attractive forces hold the molecules in contact, although they still have sufficient kinetic energy to move past each other. Intermolecular attractive forces, collectively referred to as van der Waals forces, are responsible for the behavior of liquids and solids and are electrostatic in nature. Dipole-dipole attractions result from the electrostatic attraction of the partial negative end of one dipolar molecule for the partial positive end of another. The temporary dipole that results from the motion of the electrons in an atom can induce a dipole in an adjacent atom and give rise to the London dispersion force. London forces increase with increasing molecular size. Hydrogen bonds are a special type of dipole-dipole attraction that results when hydrogen is bonded to one of the three most electronegative elements: F, O, or N.

Glossary

dipole-dipole attraction
: intermolecular attraction between two permanent dipoles

dispersion force
: (also, London dispersion force) attraction between two rapidly fluctuating, temporary dipoles; significant only when particles are very close together

hydrogen bonding
: occurs when exceptionally strong dipoles attract; bonding that exists when hydrogen is bonded to one of the three most electronegative elements: F, O, or N

induced dipole
: temporary dipole formed when the electrons of an atom or molecule are distorted by the instantaneous dipole of a neighboring atom or molecule

instantaneous dipole
: temporary dipole that occurs for a brief moment in time when the electrons of an atom or molecule are distributed asymmetrically

intermolecular force
: noncovalent attractive force between atoms, molecules, and/or ions

polarizability
: measure of the ability of a charge to distort a molecule’s charge distribution (electron cloud)

van der Waals force
: attractive or repulsive force between molecules, including dipole-dipole, dipole-induced dipole, and London dispersion forces; does not include forces due to covalent or ionic bonding, or the attraction between ions and molecules

Contributors and Attributions

- Paul Flowers (University of North Carolina - Pembroke), Klaus Theopold (University of Delaware) and Richard Langley (Stephen F. Austin State University) with contributing authors. Textbook content produced by OpenStax College is licensed under a Creative Commons Attribution License 4.0 license. Download for free at http://cnx.org/contents/85abf193-2bd...a7ac8df6@9.110).

This page titled 1.3: Intermolecular Forces is shared under a CC BY license and was authored, remixed, and/or curated by OpenStax.

- **10.2: Intermolecular Forces** by OpenStax is licensed CC BY 4.0. Original source: https://openstax.org/details/books/chemistry-2e.

1.4: Carbon and Functional Groups

Skills to Develop

- Explain why carbon is important for life
- Describe the role of functional groups in biological molecules

Cells are made of many complex molecules called macromolecules, such as proteins, nucleic acids (RNA and DNA), carbohydrates, and lipids. The macromolecules are a subset of organic molecules (any carbon-containing liquid, solid, or gas) that are especially important for life. The fundamental component for all of these macromolecules is carbon. The carbon atom has unique properties that allow it to form covalent bonds to as many as four different atoms, making this versatile element ideal to serve as the basic structural component, or "backbone," of the macromolecules.

Individual carbon atoms have an incomplete outermost electron shell. With an atomic number of 6 (six electrons and six protons), the first two electrons fill the inner shell, leaving four in the second shell. Therefore, carbon atoms can form up to four covalent bonds with other atoms to satisfy the octet rule. The methane molecule provides an example: it has the chemical formula CH_4. Each of its four hydrogen atoms forms a single covalent bond with the carbon atom by sharing a pair of electrons. This results in a filled outermost shell.

Hydrocarbons

Hydrocarbons are organic molecules consisting entirely of carbon and hydrogen, such as methane (CH_4) described above. We often use hydrocarbons in our daily lives as fuels—like the propane in a gas grill or the butane in a lighter. The many covalent bonds between the atoms in hydrocarbons store a great amount of energy, which is released when these molecules are burned (oxidized). Methane, an excellent fuel, is the simplest hydrocarbon molecule, with a central carbon atom bonded to four different hydrogen atoms, as illustrated in Figure **1.4.1**. The geometry of the methane molecule, where the atoms reside in three dimensions, is determined by the shape of its electron orbitals. The carbons and the four hydrogen atoms form a shape known as a tetrahedron, with four triangular faces; for this reason, methane is described as having tetrahedral geometry.

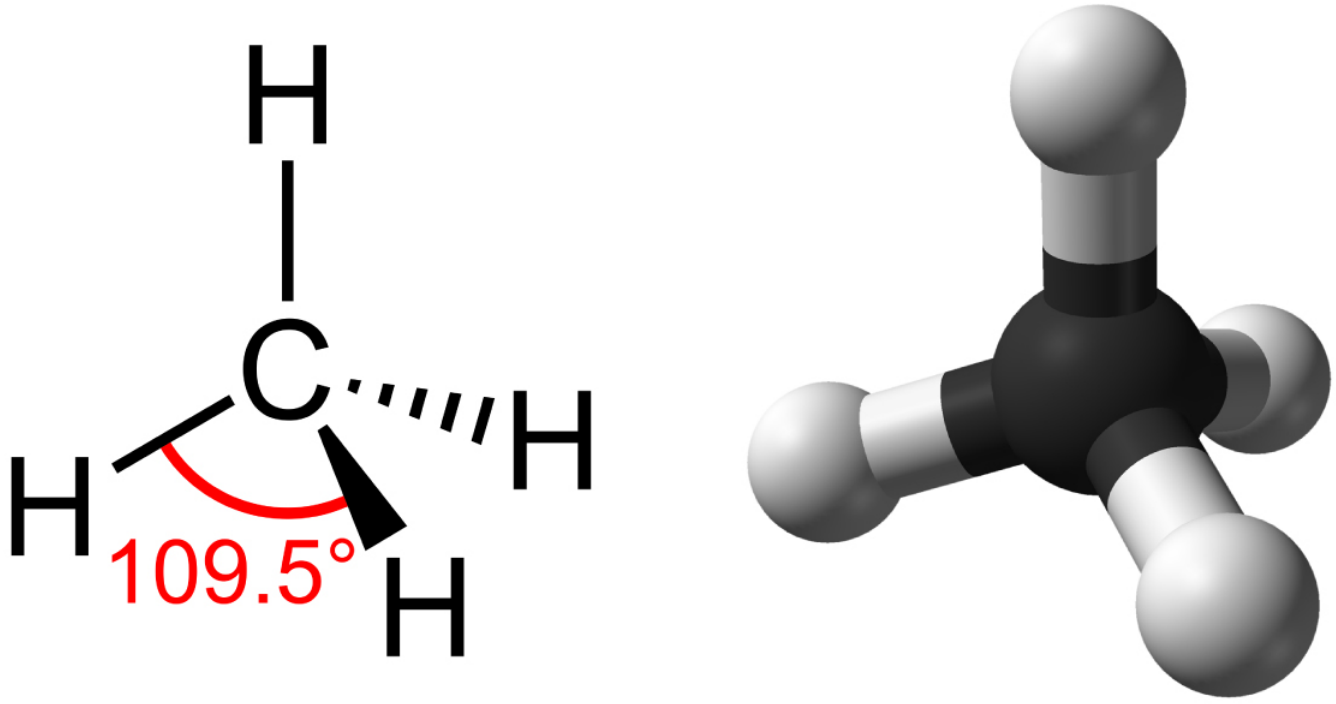


Figure **1.4.1**: Methane has a tetrahedral geometry, with each of the four hydrogen atoms spaced 109.5° apart.

As the backbone of the large molecules of living things, hydrocarbons may exist as linear carbon chains, carbon rings, or combinations of both. Furthermore, individual carbon-to-carbon bonds may be single, double, or triple covalent bonds, and each

type of bond affects the geometry of the molecule in a specific way. This three-dimensional shape or conformation of the large molecules of life (macromolecules) is critical to how they function.

Hydrocarbon Chains

Hydrocarbon chains are formed by successive bonds between carbon atoms and may be branched or unbranched. Furthermore, the overall geometry of the molecule is altered by the different geometries of single, double, and triple covalent bonds, illustrated in Figure **1.4.2**. The hydrocarbons ethane, ethene, and ethyne serve as examples of how different carbon-to-carbon bonds affect the geometry of the molecule. The names of all three molecules start with the prefix "eth-," which is the prefix for two carbon hydrocarbons. The suffixes "-ane," "-ene," and "-yne" refer to the presence of single, double, or triple carbon-carbon bonds, respectively. Thus, propane, propene, and propyne follow the same pattern with three carbon molecules, butane, butane, and butyne for four carbon molecules, and so on. Double and triple bonds change the geometry of the molecule: single bonds allow rotation along the axis of the bond, whereas double bonds lead to a planar configuration and triple bonds to a linear one. These geometries have a significant impact on the shape a particular molecule can assume.

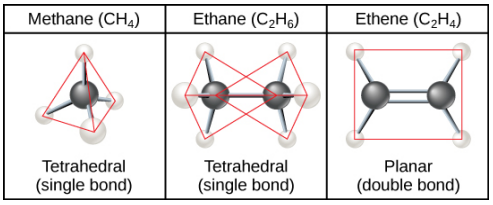


Figure **1.4.2**: When carbon forms single bonds with other atoms, the shape is tetrahedral. When two carbon atoms form a double bond, the shape is planar, or flat. Single bonds, like those found in ethane, are able to rotate. Double bonds, like those found in ethene cannot rotate, so the atoms on either side are locked in place.

Hydrocarbon Rings

So far, the hydrocarbons we have discussed have been aliphatic hydrocarbons, which consist of linear chains of carbon atoms. Another type of hydrocarbon, aromatic hydrocarbons, consists of closed rings of carbon atoms. Ring structures are found in hydrocarbons, sometimes with the presence of double bonds, which can be seen by comparing the structure of cyclohexane to benzene in Figure **1.4.3**. Examples of biological molecules that incorporate the benzene ring include some amino acids and cholesterol and its derivatives, including the hormones estrogen and testosterone. The benzene ring is also found in the herbicide 2,4-D. Benzene is a natural component of crude oil and has been classified as a carcinogen. Some hydrocarbons have both aliphatic and aromatic portions; beta-carotene is an example of such a hydrocarbon.

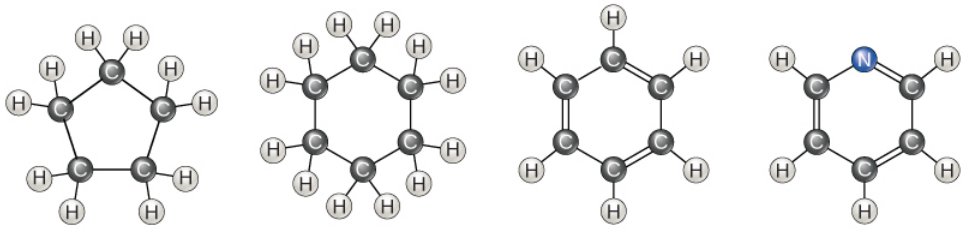


Figure **1.4.3**: Carbon can form five- and six membered rings. Single or double bonds may connect the carbons in the ring, and nitrogen may be substituted for carbon.

Isomers

The three-dimensional placement of atoms and chemical bonds within organic molecules is central to understanding their chemistry. Molecules that share the same chemical formula but differ in the placement (structure) of their atoms and/or chemical bonds are known as isomers. Structural isomers (like butane and isobutene shown in Figure **1.4.4**a differ in the placement of their covalent bonds: both molecules have four carbons and ten hydrogens (C_4H_{10}), but the different arrangement of the atoms within the molecules leads to differences in their chemical properties. For example, due to their different chemical properties, butane is suited for use as a fuel for cigarette lighters and torches, whereas isobutene is suited for use as a refrigerant and a propellant in spray cans.

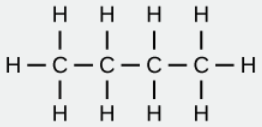
Geometric isomers, on the other hand, have similar placements of their covalent bonds but differ in how these bonds are made to the surrounding atoms, especially in carbon-to-carbon double bonds. In the simple molecule butene (C_4H_8), the two methyl groups (CH_3) can be on either side of the double covalent bond central to the molecule, as illustrated in Figure **1.4.4**b. When the carbons

are bound on the same side of the double bond, this is the *cis* configuration; if they are on opposite sides of the double bond, it is a *trans* configuration. In the *trans* configuration, the carbons form a more or less linear structure, whereas the carbons in the *cis* configuration make a bend (change in direction) of the carbon backbone.

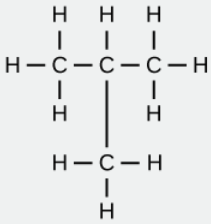
Art Connection

(a) Structural isomers

Butane

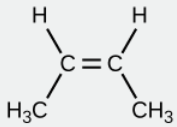


Isobutane



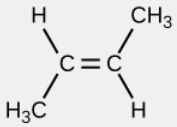
(b) Geometric isomers

cis-2-butene



methyl groups on same side of double bond

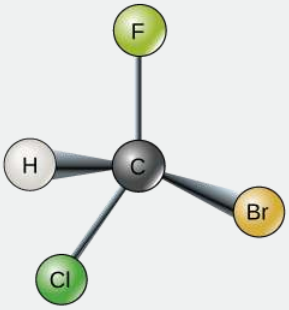
trans-2-butene



methyl groups on opposite sides of double bond

(c) Enantiomers

L-isomer



D-isomer

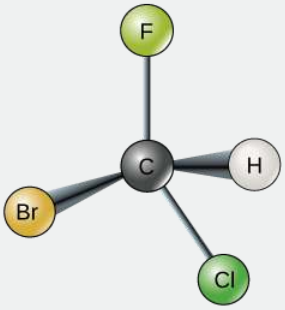


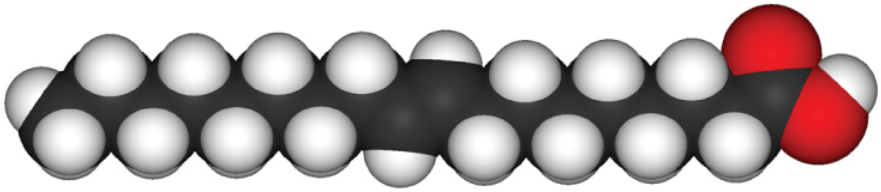
Figure **1.4.4**: Molecules that have the same number and type of atoms arranged differently are called isomers. (a) Structural isomers have a different covalent arrangement of atoms. (b) Geometric isomers have a different arrangement of atoms around a double bond. (c) Enantiomers are mirror images of each other.

Which of the following statements is false?

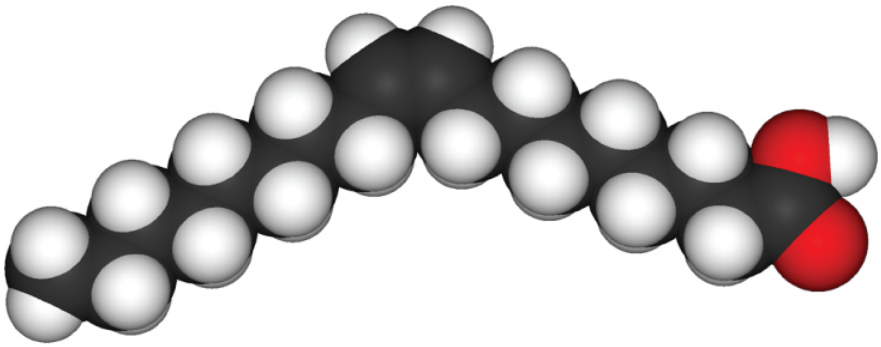
A. Molecules with the formulas CH_3CH_2COOH and $C_3H_6O_2$ could be structural isomers.
B. Molecules must have a double bond to be *cis-trans* isomers.
C. To be enantiomers, a molecule must have at least three different atoms or groups connected to a central carbon.
D. To be enantiomers, a molecule must have at least four different atoms or groups connected to a central carbon.

In triglycerides (fats and oils), long carbon chains known as fatty acids may contain double bonds, which can be in either the *cis* or *trans* configuration, illustrated in Figure **1.4.5**. Fats with at least one double bond between carbon atoms are unsaturated fats. When

some of these bonds are in the *cis* configuration, the resulting bend in the carbon backbone of the chain means that triglyceride molecules cannot pack tightly, so they remain liquid (oil) at room temperature. On the other hand, triglycerides with *trans* double bonds (popularly called trans fats), have relatively linear fatty acids that are able to pack tightly together at room temperature and form solid fats. In the human diet, trans fats are linked to an increased risk of cardiovascular disease, so many food manufacturers have reduced or eliminated their use in recent years. In contrast to unsaturated fats, triglycerides without double bonds between carbon atoms are called saturated fats, meaning that they contain all the hydrogen atoms available. Saturated fats are a solid at room temperature and usually of animal origin.



Eliadic acid



Oleic acid

Figure 1.4.5: These space-filling models show a *cis* (oleic acid) and a *trans* (eliadic acid) fatty acid. Notice the bend in the molecule cause by the *cis* configuration.

Enantiomers

Enantiomers are molecules that share the same chemical structure and chemical bonds but differ in the three-dimensional placement of atoms so that they are mirror images. As shown in Figure 1.4.6, an amino acid alanine example, the two structures are non-superimposable. In nature, only the L-forms of amino acids are used to make proteins. Some D forms of amino acids are seen in the cell walls of bacteria, but never in their proteins. Similarly, the D-form of glucose is the main product of photosynthesis and the L-form of the molecule is rarely seen in nature.

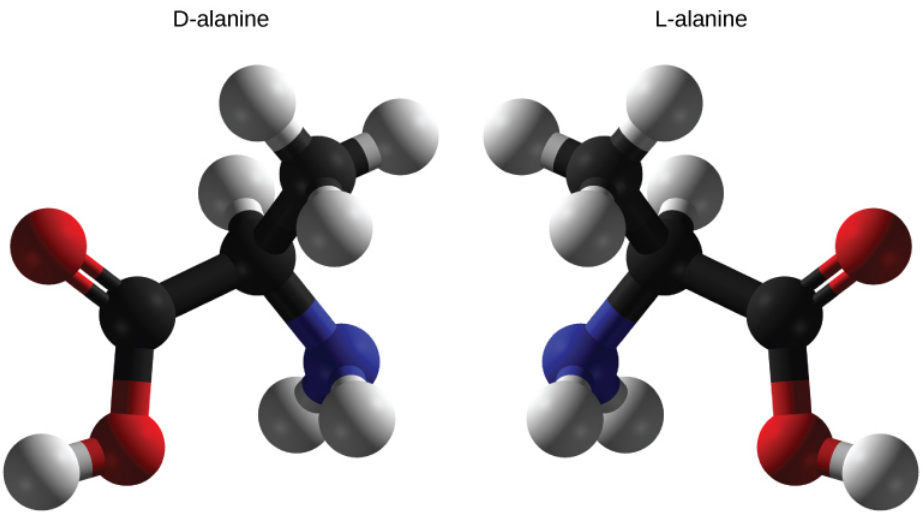


Figure 1.4.6: D-alanine and L-alanine are examples of enantiomers or mirror images. Only the L-forms of amino acids are used to make proteins.

Functional Groups

Functional groups are groups of atoms that occur within molecules and confer specific chemical properties to those molecules. They are found along the "carbon backbone" of macromolecules. This carbon backbone is formed by chains and/or rings of carbon atoms with the occasional substitution of an element such as nitrogen or oxygen. Molecules with other elements in their carbon backbone are substituted hydrocarbons.

The functional groups in a macromolecule are usually attached to the carbon backbone at one or several different places along its chain and/or ring structure. Each of the four types of macromolecules—proteins, lipids, carbohydrates, and nucleic acids—has its own characteristic set of functional groups that contributes greatly to its differing chemical properties and its function in living organisms.

A functional group can participate in specific chemical reactions. Some of the important functional groups in biological molecules are shown in Figure 1.4.7; they include: hydroxyl, methyl, carbonyl, carboxyl, amino, phosphate, and sulfhydryl. These groups play an important role in the formation of molecules like DNA, proteins, carbohydrates, and lipids. Functional groups are usually classified as hydrophobic or hydrophilic depending on their charge or polarity characteristics. An example of a hydrophobic group is the non-polar methane molecule. Among the hydrophilic functional groups is the carboxyl group found in amino acids, some amino acid side chains, and the fatty acids that form triglycerides and phospholipids. This carboxyl group ionizes to release hydrogen ions (H^+) from the COOH group resulting in the negatively charged COO^- group; this contributes to the hydrophilic nature of whatever molecule it is found on. Other functional groups, such as the carbonyl group, have a partially negatively charged oxygen atom that may form hydrogen bonds with water molecules, again making the molecule more hydrophilic.

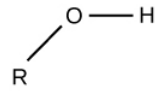
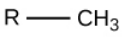
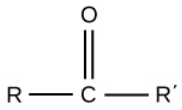
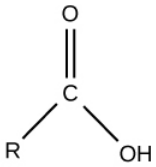
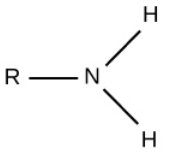
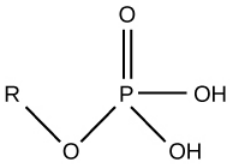
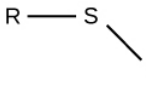
Functional Group	Structure	Properties
Hydroxyl		Polar
Methyl		Nonpolar
Carbonyl		Polar
Carboxyl		Charged, ionizes to release H^+. Since carboxyl groups can release H^+ ions into solution, they are considered acidic.
Amino		Charged, accepts H^+ to form NH_3^+. Since amino groups can remove H^+ from solution, they are considered basic.
Phosphate		Charged, ionizes to release H^+. Since phosphate groups can release H^+ ions into solution, they are considered acidic.
Sulfhydryl		Polar

Figure 1.4.7: The functional groups shown here are found in many different biological molecules.

Hydrogen bonds between functional groups (within the same molecule or between different molecules) are important to the function of many macromolecules and help them to fold properly into and maintain the appropriate shape for functioning. Hydrogen bonds are also involved in various recognition processes, such as DNA complementary base pairing and the binding of an enzyme to its substrate, as illustrated in Figure 1.4.8.

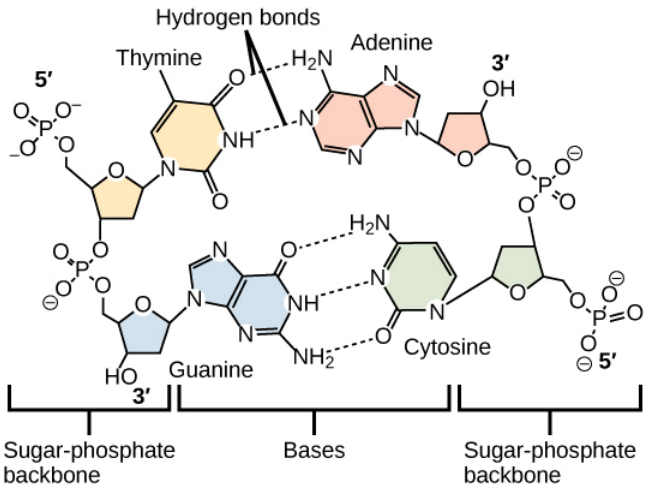


Figure 1.4.8: Hydrogen bonds connect two strands of DNA together to create the double-helix structure.

https://chem.libretexts.org/@go/page/350355

Summary

The unique properties of carbon make it a central part of biological molecules. Carbon binds to oxygen, hydrogen, and nitrogen covalently to form the many molecules important for cellular function. Carbon has four electrons in its outermost shell and can form four bonds. Carbon and hydrogen can form hydrocarbon chains or rings. Functional groups are groups of atoms that confer specific properties to hydrocarbon (or substituted hydrocarbon) chains or rings that define their overall chemical characteristics and function.

Art Connections

Figure **1.4.4**: Which of the following statements is false?

A. Molecules with the formulas CH_3CH_2COOH and $C_3H_6O_2$ could be structural isomers.
B. Molecules must have a double bond to be *cis-trans* isomers.
C. To be enantiomers, a molecule must have at least three different atoms or groups connected to a central carbon.
D. To be enantiomers, a molecule must have at least four different atoms or groups connected to a central carbon.

Answer

C

Glossary

aliphatic hydrocarbon
hydrocarbon consisting of a linear chain of carbon atoms

aromatic hydrocarbon
hydrocarbon consisting of closed rings of carbon atoms

enantiomers
molecules that share overall structure and bonding patterns, but differ in how the atoms are three dimensionally placed such that they are mirror images of each other

functional group
group of atoms that provides or imparts a specific function to a carbon skeleton

geometric isomer
isomer with similar bonding patterns differing in the placement of atoms alongside a double covalent bond

hydrocarbon
molecule that consists only of carbon and hydrogen

isomers
molecules that differ from one another even though they share the same chemical formula

organic molecule
any molecule containing carbon (except carbon dioxide)

structural isomers
molecules that share a chemical formula but differ in the placement of their chemical bonds

substituted hydrocarbon
hydrocarbon chain or ring containing an atom of another element in place of one of the backbone carbons

This page titled 1.4: Carbon and Functional Groups is shared under a CC BY license and was authored, remixed, and/or curated by OpenStax.

- **2.3: Carbon** by OpenStax is licensed CC BY 4.0.

1.5: Water, Equilibrium, and Buffers

Source: BiochemFFA_1_3.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy



When it comes to water, we're literally drowning in it, as water is by far the most abundant component of every cell. To understand life, we begin the discussion with the basics of water, because everything that happens in cells, even reactions buried deep inside enzymes, away from water, is influenced by water's chemistry.

The water molecule has wide 'V' shape (the HO-H angle is 104°) with uneven sharing of electrons between the oxygen and the hydrogen atoms (Figure 1.23). Oxygen, with its higher electronegativity, holds electrons closer to itself than the hydrogens do. The hydrogens, as a result, are described as having a partial positive charge (typically designated as δ+) and the oxygen has a partial negative charge (written as δ-). Thus, water is a polar molecule because charges are distributed around it unevenly, not symmetrically.

Water as a solvent

Water (Figure 1.23) is described as a solvent because of its ability to solvate (dissolve) many, but not all, molecules. Molecules that are ionic or polar dissolve readily in water, but non-polar substances dissolve poorly in water, if at all. Oil, for example, which is non-polar, separates from water when mixed with it. On the other hand, sodium chloride, which ionizes, and ethanol, which is polar, are able to form hydrogen bonds, so both dissolve in water. Ethanol's solubility in water is crucial for brewers, winemakers, and distillers – but for this property, there would be no wine, beer or spirits. As explained in an earlier section, we use the term hydrophilic to describe substances that interact well with water and dissolve in it and the term hydrophobic to refer to materials that are non-polar and do not dissolve in water. Table 1.3 illustrates some polar and non-polar substances. A third term, amphiphilic, refers to compounds that have both properties. Soaps, for example are amphiphilic, containing a long, non-polar aliphatic tail and a head that ionizes.

Hydrophilic vs Hydrophobic Compounds

Hydrophobic	Hydrophilic
Nonpolar hydrocarbons (hexane)	Ionic compounds (NaCl)
Lipids (fats and cholesterol)	Polar organic compounds (alcohols, ketones or carbonyls)
	Weak acids (phosphates, amino acids)
	Sugars/carbohydrates

Table 1.3 Image by Aleia Kim

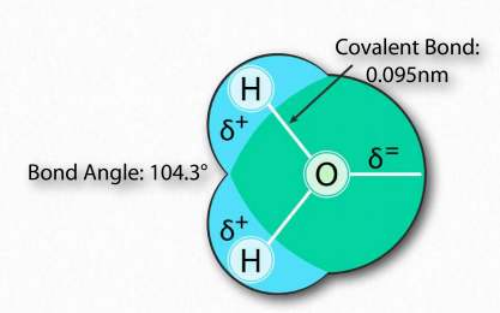


Figure 1.23 - Arrangement of atoms in water Image by Aleia Kim

The solubility of materials in water is based in free energy changes, as measured by ΔG. Remember, from chemistry, that H is the enthalpy (heat at constant pressure) and S is entropy. Given this,

$$\Delta G = \Delta H - T\Delta S \tag{1.5.1}$$

where T is the temperature in Kelvin. For a process to be favorable, the ΔG for it must be less than zero.

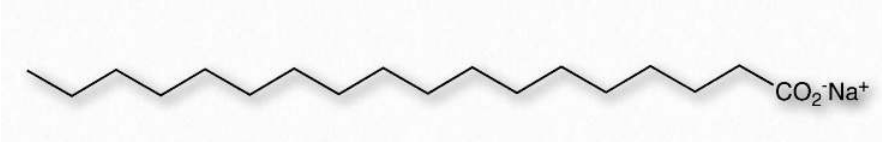


Figure 1.24 - Structure of a Soap

From the equation, lowered ΔG values will be favored with decreases in enthalpy and/or increases in entropy. Let us first consider why non-polar materials do not dissolve in water. We could imagine a situation where the process of dissolving involves the "surrounding" of each molecule of the nonpolar solute in water, just like each sodium and each chloride ion gets surrounded by water molecules as salt dissolves.

Water organization

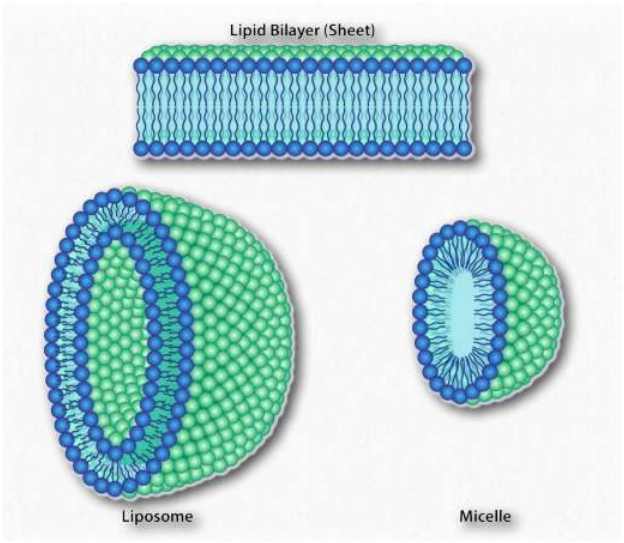


Figure 1.25 - Structures formed by amphiphilic substances in water. Image by Aleia Kim

There is a significant difference, though between surrounding a non-polar molecule with water molecules and surrounding ions (or polar compounds) with water molecules.

The difference is that since non-polar molecules don't really interact with water, the water behaves very differently than it does with ions or molecules that form hydrogen bonds. In fact, around each non-polar molecule, water gets very organized, aligning itself regularly. As any freshman chemistry student probably remembers, entropy is a measure of disorder, so when something

becomes ordered, entropy decreases, meaning the ΔS is negative, so the TΔS term in the equation is positive (negative of a negative).

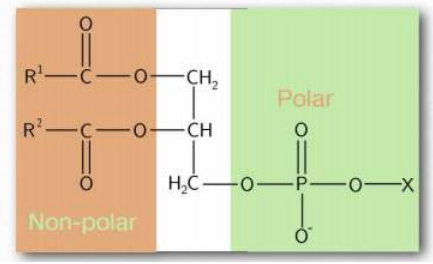


Figure 1.26 - A phospholipid - an amphiphilic substance

Since mixing a non-polar substance with water doesn't generally have any significant heat component, the ΔG is positive. This means, then, that dissolving a non-polar compound in water is not favorable and does not occur to any significant extent. Further, when the non-polar material associates with itself and not water, then the water molecules are free to mix, without being ordered, resulting in an increase of entropy. Entropy therefore drives the separation of non-polar substances from aqueous solutions.

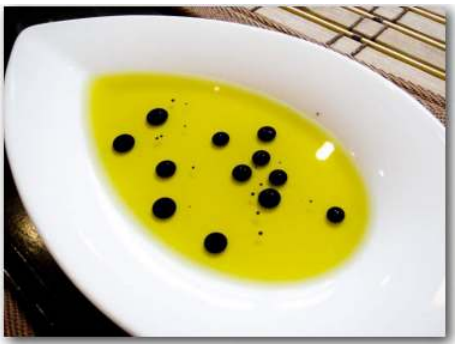


Figure 1.27 - Vinegar (black) and oil (yellow) A mix of polar and nonpolar compounds Wikipedia

Amphiphilic substances

Next, we consider mixing of an amphiphilic substance, such as a soap, with water (Figure 1.24). The sodium ions attached to the fatty acids in soap readily come off in aqueous solution, leaving behind a negatively charged molecule at one end and a non-polar region at the other end. The ionization of the soap causes in an increase in entropy - two particles instead of one. The non-polar portion of the negatively charged soap ion is problematic - if exposed to water, it will cause water to organize and result in a decrease of entropy and a positive ΔG.

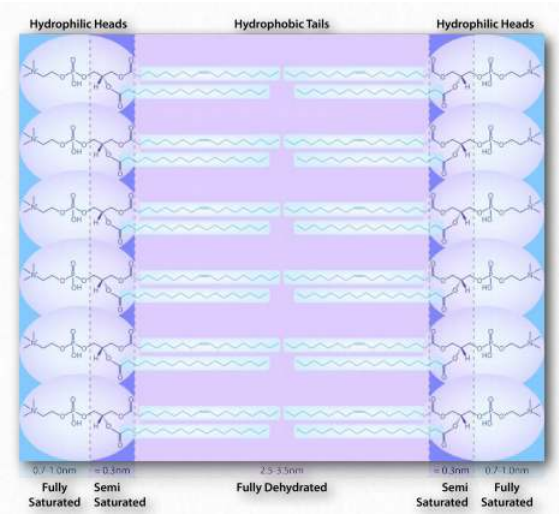


Figure 1.28 - Environment of a lipid bilayer. Water is concentrated away from the hydrophobic center, being saturated on the outside, semi-saturated near the head-tail junction and fully dehydrated in the middle. Image by Aleia Kim

Since we know fatty acids dissolve in water, there must be something else at play. There is. Just like the non-polar molecules in the first example associated with each other and not water, so too do the non-polar portions of the soap ions associate with each other and exclude water. The result is that the soap ions arrange themselves as micelles (Figure 1.25) with the non-polar portions on the interior of the structure away from water and the polar portions on the outside interacting with water.

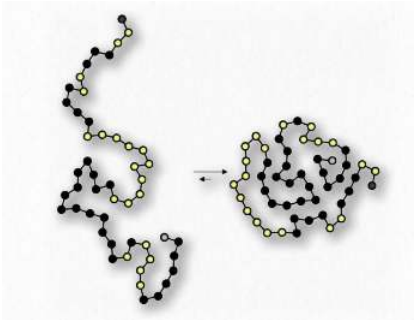


Figure 1.29 - Protein folding arranges hydrophobic amino acids (black dots) inside the protein

The interaction of the polar heads with water returns the water to its more disordered state. This increase in disorder, or entropy, drives the formation of micelles. As will be seen in the discussion of the lipid bilayer, the same forces drive glycerophospholipids and sphingolipids to spontaneously form bilayers where the non-polar portions of the molecules interact with each other to exclude water and the polar portions arrange themselves on the outsides of the bilayer (Figure 1.28).

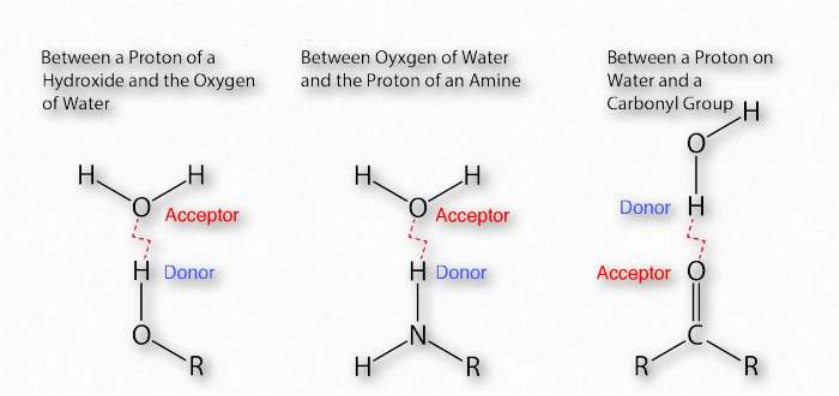


Figure 1.30 - Common hydrogen bonds in biochemistry Image by Aleia Kim

Yet another example is seen in the folding of globular proteins in the cytoplasm. Nonpolar amino acids are found in the interior portion of the protein (water excluded). Interaction of the non-polar amino acids turns out to be a driving force for the folding of proteins as they are being made in an aqueous solution.

Hydrogen bonds

The importance of hydrogen bonds in biochemistry (Figure 1.30) is hard to overstate. Linus Pauling himself said,

" . . . . I believe that as the methods of structural chemistry are further applied to physiological problems it will be found that the significance of the hydrogen bond for physiology is greater than that of any other single structural feature."

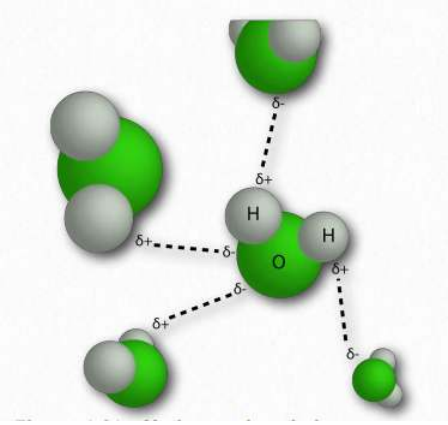


Figure 1.31 - Hydrogen bonds between water molecules Image by Pehr Jacobson

In 2011, an IUPAC task group gave an evidence-based definition of hydrogen bonding that states,

"The hydrogen bond is an attractive interaction between a hydrogen atom from a molecule or a molecular fragment X–H in which X is more electronegative than H, and an atom or a group of atoms in the same or a different molecule, in which there is evidence of bond formation."

Partial Charges

The difference in electronegativity between hydrogen and the molecule to which it is covalently bound give rise to partial charges as described above. These tiny charges (δ+ and δ-) result in formation of hydrogen bonds, which occur when the partial positive charge of a hydrogen atom is attracted to the partial negative of another molecule. In water, that means the hydrogen of one water molecule is attracted to the oxygen of another (Figure 1.31). Since water is an asymmetrical molecule, it means also that the charges are asymmetrical. Such an uneven distribution is what makes a dipole. Dipolar molecules are important for interactions with other dipolar molecules and for dissolving ionic substances (Figure 1.32).

Hydrogen bonds are not exclusive to water. In fact, they are important forces holding together macromolecules that include proteins and nucleic acids. Hydrogen bonds occur within and between macromolecules.

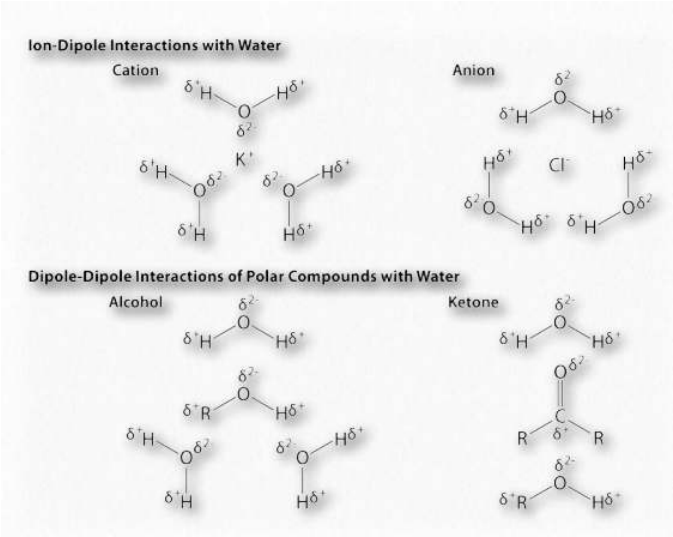


Figure 1.32 - Example dipole interactions in biochemistry Image by Aleia Kim

The complementary pairing that occurs between bases in opposite strands of DNA, for example, is based on hydrogen bonds. Each hydrogen bond is relatively weak (compared to a covalent bond, for example - Table 1.4), but collectively they can be quite strong.

Bond Energies		
	Type of Bond	Bond Energy (kJ/mol)
Covalent Bonds	C—H	413
	O—H	460
Noncovalent Bonds	Hydrophobic Interaction	4-12
	Hydrogen Bond	20
	Ion-dipole Interaction	20

Table 1.4 Image by Aleia Kim

Benefits of weak interactions

Their weakness, however, is actually quite beneficial for cells, particularly as regards nucleic acids (Figure 1.33). The strands of DNA, for example, must be separated over short stretches in the processes of replication and the synthesis of RNA. Since only a few base pairs at a time need to be separated, the energy required to do this is small and the enzymes involved in the processes can readily take them apart, as needed. Hydrogen bonds also play roles in binding of substrates to enzymes, catalysis, and protein-protein interaction, as well as other kinds of binding, such as protein-DNA, or antibody-antigen.

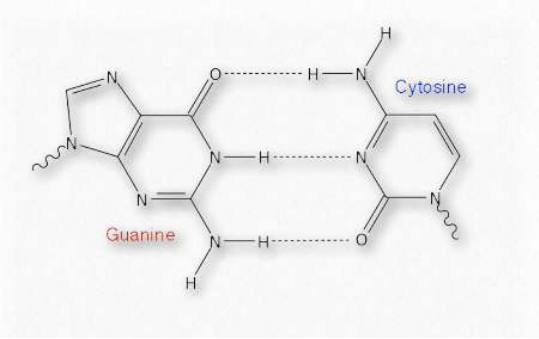


Figure 1.33 - Hydrogen bonds in a base pair of DNA Image by Aleia Kim

As noted, hydrogen bonds are weaker than covalent bonds (Table 1.4) and their strength varies form very weak (1-2 kJ/mol) to fairly strong (29 kJ/mol). Hydrogen bonds only occur over relatively short distances (2.2 to 4.0 Å). The farther apart the hydrogen bond distance is, the weaker the bond is.

The strength of the bond in kJ/mol represents the amount of heat that must be put into the system to break the bond - the larger the number, the greater the strength of the bond. Hydrogen bonds are readily broken using heat. The boiling of water, for example, requires breaking of H-bonds. When a biological structure, such as a protein or a DNA molecule, is stabilized by hydrogen bonds, breaking those bonds destabilizes the structure and can result in denaturation of the substance - loss of structure. It is partly for this reason that most proteins and all DNAs lose their native, or folded, structures when heated to boiling.

Weak Acid pKa Values

Name	Chemical Structure of Acid	Chemical Structure of Salt	pKa
Acetic Acid	CH_3COOH	CH_3COO^-	4.76
Formic Acid	$HCOOH$	$HCOO^-$	3.75
Lactic Acid	$CH_3CHOHCOOH$	$CH_3CH—HCOO^-$	3.86
Pyruvic Acid	$CH_3COCOOH$	$CH_3C—COO^-$	2.50
Oxalic Acid (1)	$HOOC—COOH$	$HOOC—COO^-$	1.23
Oxalic Acid (2)	$HOOC—COO^-$	$OOC—COO^-$	4.19
Carbonic Acid (1)	H_2CO_3	HCO_3^-	6.37
Carbonic Acid (2)	HCO_3^-	CO_3^{2-}	10.20
Malic Acid (1)	$HOOC—CH_2—CHOH—COOH$	$HOOC—CH_2—CHOH—COO^-$	3.40
Malic Acid (2)	$HOOC—CH_2—CHOH—COO^-$	$^-OOC—CH_2—CHOH—COO^-$	5.26
Malonic Acid (1)	$HOOC—CH_2—COOH$	$HOOC—CH_2—COO^-$	2.83
Malonic Acid (2)	$HOOC—CH_2—COO^-$	$^-OOC—CH_2—COO^-$	5.69
Phosphoric Acid (1)	H_3PO_4	$H_2PO_4^-$	2.14
Phosphoric Acid (2)	$H_2PO_4^-$	HPO_4^{2-}	7.20
Phosphoric Acid (3)	HPO_4^{2-}	PO_4^{3-}	12.40
Succinic Acid (1)	$HOOC—CH_2—CH_2—COOH$	$HOOC—CH_2—CH_2—COO^-$	4.21
Succinic Acid (2)	$HOOC—CH_2—CH_2—COO^-$	$^-OOC—CH_2—CH_2—COO^-$	5.63

Image by Aleia Kim Table 1.5

For DNA molecules, denaturation results in complete separation of the strands from each other. For most proteins, this means loss of their characteristic three-dimensional structure and with it, loss of the function they performed. Though a few proteins can readily reassume their original structure when the solution they are in is cooled, most can't. This is one of the reasons that we cook our food. Proteins are essential for life, so denaturation of bacterial proteins results in death of any microorganisms contaminating the food.

The importance of buffers

Water can ionize to a slight extent (10-7 M) to form H+ (proton) and OH- (hydroxide). We measure the proton concentration of a solution with pH, which is the negative log of the proton concentration.

$$pH = -Log[H+]$$

If the proton concentration, [H+]= 10-7 M, then the pH is 7. We could just as easily measure the hydroxide concentration with the pOH by the parallel equation,

$$pOH = -Log[OH^-]$$

In pure water, dissociation of a proton simultaneously creates a hydroxide, so the pOH of pure water is 7, as well. This also means that

$$pH + pOH = 14$$

Now, because protons and hydroxides can combine to form water, a large amount of one will cause there to be a small amount of the other. Why is this the case? In simple terms, if I dump 0.1 moles of H+ into a pure water solution, the high proton concentration will react with the relatively small amount of hydroxides to create water, thus reducing hydroxide concentration. Similarly, if I dump excess hydroxide (as NaOH, for example) into pure water, the proton concentration falls for the same reason.

Acids vs bases

Chemists use the term "acid" to refer to a substance which has protons that can dissociate (come off) when dissolved in water. They use the term "base" to refer to a substance that can absorb protons when dissolved in water. Both acids and bases come in strong and weak forms. (Examples of weak acids are shown in Table 1.5.) Strong acids, such as HCl, dissociate completely in water. If we add 0.1 moles (6.02x1022 molecules) of HCl to a solution to make a liter, it will have 0.1 moles of H+ and 0.1 moles of Cl- or

6.02x1022 molecules of each . There will be no remaining HCl when this happens. A strong base like NaOH also dissociates completely into Na+ and OH- .

Clearing Confusion - Students are often puzzled and expect that

$$[H^+] = [A^-]$$

because the dissociation equation shows one of each from HA. This is, in fact, true ONLY when HA is allowed to dissociate in pure water. Usually the HA is placed into solution that has protons and hydroxides to affect things. Those protons and /or hydroxides change the H^+ and A^- concentration unequally, since A^- can absorb some of the protons and/or HA can release H^+ when influenced by the OH^- in the solution. Therefore, one must calculate the proton concentration from the pH using the Henderson Hasselbalch equation.

$$pH = pK_a + \log([Ac^-]/[HAc])$$

Weak Acids

Weak acids and bases differ from their strong counterparts. When you put one mole of acetic acid (HAc) into pure water, only a tiny percentage of the HAc molecules dissociate into H+ and Ac- . Clearly, weak acids are very different from strong acids. Weak bases behave similarly, except that they accept protons, rather than donate them. Since we can view everything as a form of a weak acid, we will not use the term weak base here.

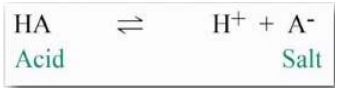


Figure 1.34 - Dissociation of a weak acid Image by Aleia Kim

Students are often puzzled and expect that $[H^+] = [A^-]$ because the dissociation equation shows one of each from HA. This is, in fact, true ONLY when HA is allowed to dissociate in pure water. Usually the HA is placed into solution that has protons and hydroxides to affect things. Those protons and /or hydroxides change the H+ and Aconcentration unequally, since A- can absorb some of the protons and/or HA can release H+ when influenced by the OH- in the solution. Therefore, one must calculate the proton concentration from the pH using the Henderson Hasselbalch equation.

$$pH = pKa + \log([Ac^-]/[HAc])$$

Salt/Acid Ratio as a Function of pK_as

pH	[Salt]/[Acid]
$pK_a + 3$	1000
$pK_a + 2$	100
$pK_a + 1$	10
pK_a	1
$pK_a - 1$	1/10
$pK_a - 2$	1/100
$pK_a - 3$	1/1000

Image by Aleia Kim Table 1.6

You may wonder why we care about weak acids. You may never have thought much of weak acids when you were in General Chemistry. Your instructor described them as buffers and you probably dutifully memorized the fact that “buffers are substances that resist change in pH” without really learning what Clearing Confusion - this meant. Buffers are much too important to be thought of in this way.

UPS

Weak acids are critical for life because their affinity for protons causes them to behave like a UPS. We're not referring to the UPS that is the United Parcel Service, but instead, to the encased battery backup systems for computers called Uninterruptible Power Supplies that kick on to keep a computer running during a power failure. The battery in a laptop computer is a UPS, for example.

We can think of weak acids as Uninterruptible Proton Suppliers within certain pH ranges, providing (or absorbing) protons as needed. Weak acids thus help to keep the H+ concentration (and thus the pH) of the solution they are in relatively constant.

Consider the bicarbonate/carbonic acid system. Figure 1.35 shows what happens when H_2CO_3dissociates. Adding hydroxide ions (by adding a strong base like NaOH) to the solution causes the H+ ions to react with OH- ions to make water. Consequently, the concentration of H+ ions would go down and the pH would go up.

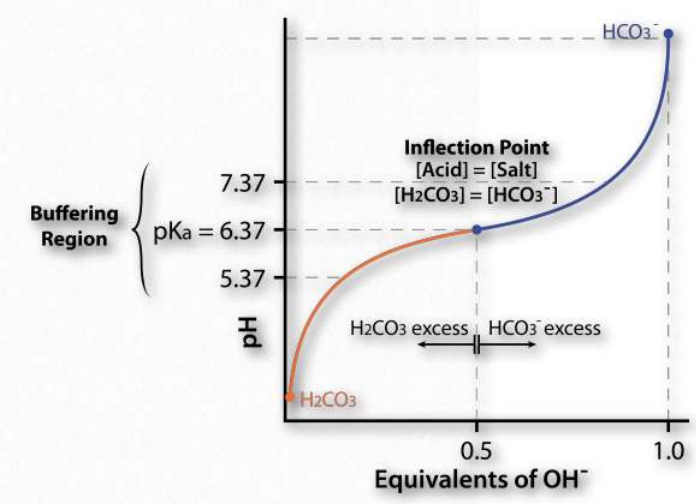


Figure 1.35 - Titration curve for carbonic acid Image by Aleia Kim

However, in contrast to the situation with a solution of pure water, there is a backup source of H+ available in the form of H_2CO_3. Here is where the UPS function kicks in. As protons are taken away by the added hydroxyl ions (making water), they are partly replaced by protons from the H_2CO_3. This is why a weak acid is a buffer. It resists changes in pH by releasing protons to compensate for those "used up" in reacting with the hydroxyl ions.

Why do we care about pH? Because biological molecules can, in some cases, be exquisitely sensitive to changes in it. As the pH of a solution changes, the charges of molecules in the solution can change, as you will see. Changing charges on biological molecules, especially proteins, can drastically affect how they work and even whether they work at all.

Henderson-Hasselbalch

It is useful to be able to predict the response of the H_2CO_3 system to changes in H+ concentration. The Henderson-Hasselbalch equation defines the relationship between pH and the ratio of HCO_3^- and H_2CO_3. It is

$$pH = pKa + \log ([HCO_3^-]/ [H_2CO_3])$$

This simple equation defines the relationship between the pH of a solution and the ratio of HCO_3^- and H_2CO_3 in it. The new term, called the pKa, is defined as

$$pKa = -\text{Log } K_a,$$

just as

$$pH = -\text{Log } [H^+].$$

The Ka is the acid dissociation constant and is a measure of the strength of an acid. For a general acid, HA, which dissociates as

$$HA \rightleftarrows H^+ + A^-,\ K_a = [H^+][A^-]/[HA]$$

Thus, the stronger the acid, the more protons that will dissociate from it when added to water and the larger the value its Ka will have. Large values of Ka translate to lower values of pKa. As a result, the lower the pKa value is for a given acid, the stronger the weak acid is.

Constant pKa

Please note that pKa is a constant for a given acid. The pKa for carbonic acid is 6.37. By comparison, the pKa for formic acid is 3.75. Formic acid is therefore a stronger acid than acetic acid. A stronger acid will have more protons dissociated at a given pH than a weaker acid.

Now, how does this translate into stabilizing pH? Figure 1.35 shows a titration curve. In this curve, the titration begins with the conditions at the lower left (very low pH). At this pH, the H_2CO_3 form predominates, but as more and more OH- is added (moving to the 45 Why do we care about pH? Because biological molecules can, in some cases, be exquisitely sensitive to changes in it. As the pH of a solution changes, the charges of molecules in the solution can change, as you will see. Changing charges on biological molecules, especially proteins, can drastically affect how they work and even whether they work at all right), the pH goes up, the amount of HCO3- goes up and (correspondingly), the amount of H_2CO_3 goes down. Notice that the curve “flattens” near the pKa (6.37).

Buffering region

Flattening of the curve tells us is that the pH is not changing much (not going up as fast) as it did earlier when the same amount of hydroxide was added. The system is resisting a change in pH (not stopping the change, but slowing it) in the region of about one pH unit above and one pH unit below the pKa. Thus, the buffering region of the carbonic acid/ bicarbonate buffer is from about 5.37 to 7.37. It is maximally strong at a pH of 6.37.

Now it starts to become apparent how the buffer works. HA can donate protons when extras are needed (such as when OH- is added to the solution by the addition of NaOH). Similarly, A- can accept protons when extra H+ are added to the solution (adding HCl, for example). The maximum ability to donate or accept protons comes when

$$[A^-] = [HA]$$

This is consistent with the Henderson Hasselbalch equation and the titration curve. When $[A^-] = [HA]$, pH = 6.37 + Log(1). Since Log(1) = 0, pH = 6.37 = pKa for carbonic acid. Thus for any buffer, the buffer will have maximum strength and display flattening of its titration curve when $[A^-] = [HA]$ and when pH = pKa. If a buffer has more than one pKa (Figure 1.36), then each pKa region will display the behavior.

Buffered vs non-buffered

To understand how well a buffer protects against changes in pH, consider the effect of adding .01 moles of HCl to 1.0 liter of pure water (no volume change) at pH 7, compared to adding it to 1.0 liter of a 1M acetate buffer at pH 4.76. Since HCl completely dissociates, in 0.01M (10-2 M) HCl you will have 0.01M H+. For the pure water, the pH drops from 7.0 down to 2.0 (pH = -log(0.01M)).

By contrast, the acetate buffer’s pH after adding the same amount of HCl is 4.74. Thus, the pure water solution sees its pH fall from 7 to 2 (5 pH units), whereas the buffered solution saw its pH drop from 4.76 to 4.74 (0.02 pH units). Clearly, the buffer minimizes the impact of the added protons compared to the pure water.

Buffer capacity

It is important to note that buffers have capacities limited by their concentration. Let’s imagine that in the previous paragraph, we had added the 0.01 moles HCl to an acetate buffer that had a concentration of 0.01M and equal amounts of Ac- and HAc. When we try to do the math in parallel to the previous calculation, we see that there are 0.01M protons, but only 0.005M A- to absorb them. We could imagine that 0.005M of the protons would be absorbed, but that would still leave 0.005M of protons unbuffered. Thus, the pH of this solution would be approximately

$$pH = -\log(0.005M) = 2.30$$

Exceeding buffer capacity dropped the pH significantly compared to adding the same amount of protons to a 1M acetate buffer. Consequently, when considering buffers, it is important to recognize that their concentration sets their limits. Another limit is the pH range in which one hopes to control proton concentration.

Multiple ionizable groups

Now, what happens if a molecule has two (or more) ionizable groups? It turns out, not surprisingly, that each group will have its own pKa and, as a consequence, will have multiple regions of buffering.

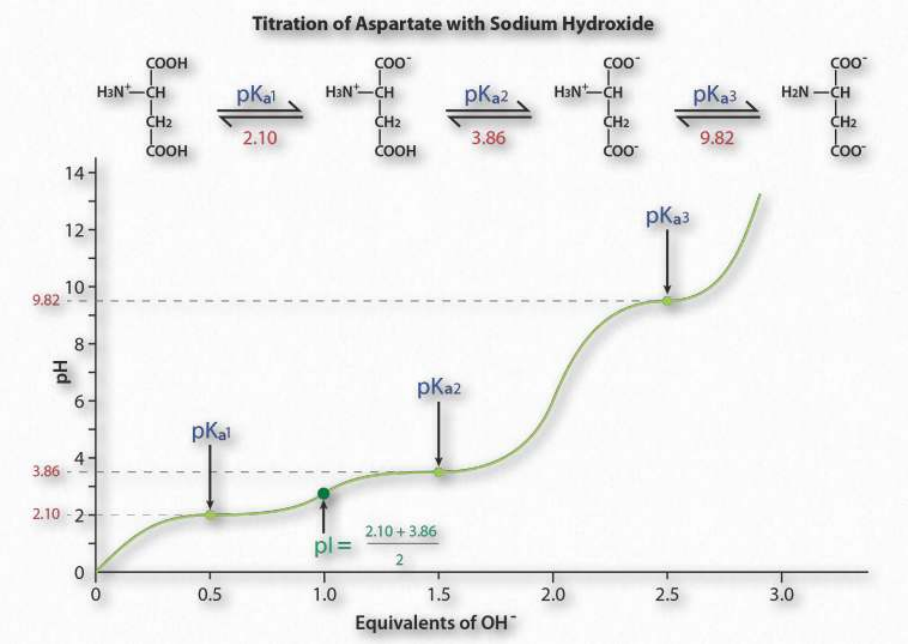


Figure 1.36 - Titration of an acidic amino acid Image by Aleia Kim

Figure 1.36 shows the titration curve for the amino acid aspartic acid. Note that in- stead of a single flattening of the curve, as was seen for acetic acid, aspartic acid's titration curve displays three such regions. These are individual buffering regions, each centered on the respective pKa values for the carboxyl group and the amine group.

Aspartic acid has four possible charges: +1 (α-carboxyl group, α-amino group, and Rgroup carboxyl each has a proton), 0 (α-carboxyl group missing proton, α- amino group has a proton, R-group carboxyl has a proton), -1 (α-carboxyl group and R-group carboxyl each lack a proton, α-amino group retains a proton), -2 (α-carboxyl, R-group carboxyl, and α-amino groups all lack extra proton).

Prediction

How does one predict the charge for an amino acid at a given pH? A good rule of thumb for estimating charge is that if the pH is more than one unit below the pKa for a group (carboxyl or amino), the proton is on. If the pH is more than one unit above the pKa for the group, the proton is off. If the pH is NOT more than one or less than one pH unit from the pKa, this simple assumption will not work.

Further, it is important to recognize that these rules of thumb are estimates only. The pI (pH at which the charge of a molecule is zero) is an exact value calculated as the average of the two pKa values on either side of the zero region. It is calculated at the average of the two pKa values around the point where the charge of the molecule is zero. For aspartic acid, this corresponds to $pK_{a1}$and pK_{a2}.

References

1. http://www.lpi.usra.edu/lunar/missions/apollo/ apollo_12/experiments/surveyor/
2. Arunan, Elangannan; Desiraju, Gautam R.; Klein, Roger A.; Sadlej, Joanna; Scheiner, Steve; Alkorta, Ibon; Clary, David C.; Crabtree, Robert H.; Dannenberg, Joseph J.; Hobza, Pavel; Kjaergaard, Henrik G.; Legon, Anthony C.; Mennucci, Benedetta;

Nesbitt, David J. (2011). "Definition of the hydrogen bond". Pure Appl. Chem. 83 (8): 1637–1641. doi:10.1351/PAC-REC-10-01-02

This page titled 1.5: Water, Equilibrium, and Buffers is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **1.3: Introduction - Water and Buffers** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

CHAPTER OVERVIEW

2: Structure and Function- Proteins

2.1: Structure and Function - Amino Acids

2.2: Structure and Function- Proteins I

2.3: Structure and Function- Proteins II

2: Structure and Function- Proteins is shared under a not declared license and was authored, remixed, and/or curated by LibreTexts.

2.1: Structure and Function - Amino Acids

Source: BiochemFFA_2_1.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

All of the proteins on the face of the earth are made up of the same 20 amino acids. Linked together in long chains called polypeptides, amino acids are the building blocks for the vast assortment of proteins found in all living cells.



"It is one of the more striking generalizations of biochemistry ...that the twenty amino acids and the four bases, are, with minor reservations, the same throughout Nature." - Francis Crick

All amino acids have the same basic structure, which is shown in Figure 2.1. At the “center” of each amino acid is a carbon called the α carbon and attached to it are four groups - a hydrogen, an α- carboxyl group, an α-amine group, and an R-group, sometimes referred to as a side chain. The α carbon, carboxyl, and amino groups are common to all amino acids, so the R-group is the only unique feature in each amino acid. (A minor exception to this structure is that of proline, in which the end of the R-group is attached to the α-amine.) With the exception of glycine, which has an R-group consisting of a hydrogen atom, all of the amino acids in proteins have four different groups attached to them and consequently can exist in two mirror image forms, L and D. With only very minor exceptions, every amino acid found in cells and in proteins is in the L configuration.

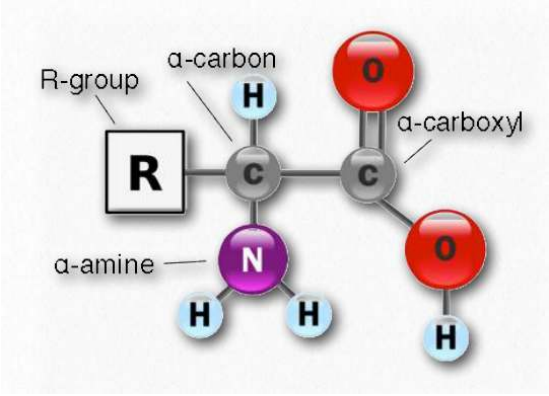


Figure 2.1 - General amino acid structure

There are 22 amino acids that are found in proteins and of these, only 20 are specified by the universal genetic code. The others, selenocysteine and pyrrolysine use tRNAs that are able to base-pair with stop codons in the mRNA during translation. When this happens, these unusual amino acids can be incorporated into proteins. Enzymes containing selenocysteine, for example, include glutathione peroxidases, antioxidant enzymes that reduce peroxide to water and limit potential oxidative damage peroxide can cause to proteins, DNA and lipids. Pyrrolysine-containing proteins are much rarer and are mostly confined to archaea.

Essential and non-essential amino acids

Nutritionists divide amino acids into two groups - essential amino acids (must be in the diet because cells can't synthesize them) and non-essential amino acids (can be made by cells). This classification of amino acids has little to do with the structure of amino acids. Essential amino acids vary considerable from one organism to another and even differ in humans, depending on whether they are adults or children. Table 2.1 shows essential and non-essential amino acids in humans.

Some amino acids that are normally nonessential, may need to be obtained from the diet in certain cases. Individuals who do not synthesize sufficient amounts of arginine, cysteine, glutamine, proline, selenocysteine, serine, and tyrosine, due to illness, for example, may need dietary supplements containing these amino acids.

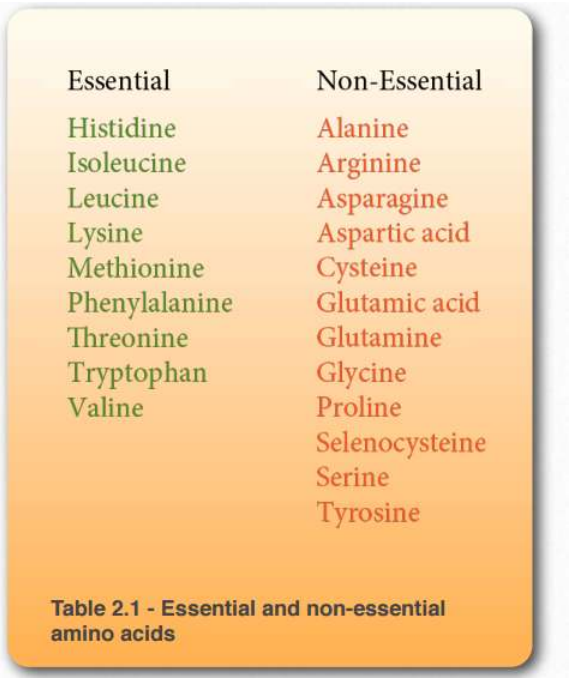


Table 2.1 - Essential and non-essential amino acids

Non-protein amino acids

There are also α-amino acids found in cells that are not incorporated into proteins. Common ones include ornithine and citrulline. Both of these compounds are intermediates in the urea cycle. Ornithine is a metabolic precursor of arginine and citrulline can be produced by the breakdown of arginine. The latter reaction produces nitric oxide, an important signaling molecule. Citrulline is a metabolic byproduct. It is sometimes used as a dietary supplement to reduce muscle fatigue.

R-group chemistry

Non-Polar	Carboxyl	Amine	Aromatic	Hydroxyl	Other
Alanine	Aspartic Acid	Arginine	Phenylalanine	Serine	Asparagine
Glycine	Glutamic Acid	Histidine	Tryptophan	Threonine	Cysteine
Isoleucine		Lysine	Tyrosine	Tyrosine	Glutamine
Leucine					Selenocysteine
Methionine					Pyrrolysine
Proline					
Valine					

Table 2.2 - Amino acid categories (based on R-group properties)

We separate the amino acids into categories based on the chemistry of their R-groups. If you compare groupings of amino acids in different textbooks, you will see different names for the categories and (sometimes) the same amino acid being categorized differently by different authors. Indeed, we categorize tyrosine both as an aromatic amino acid and as a hydroxyl amino acid. It is useful to classify amino acids based on their R-groups, because it is these side chains that give each amino acid its characteristic properties. Thus, amino acids with (chemically) similar side groups can be expected to function in similar ways, for example, during protein folding.

Non-polar amino acids

- Alanine (Ala/A) is one of the most abundant amino acids found in proteins, ranking second only to leucine in occurrence. A D-form of the amino acid is also found in bacterial cell walls. Alanine is non-essential, being readily synthesized from pyruvate.
- Glycine (Gly/G) is the amino acid with the shortest side chain, having an R-group consistent only of a single hydrogen. As a result, glycine is the only amino acid that is not chiral. Its small side chain allows it to readily fit into both hydrophobic and hydrophilic environments.

Amino acid	Short	Abbrev.	Side chain	Hydro-phobic	pKa	Polar	pH	Small	Tiny	Aromatic or Aliphatic	van der Waals volume
Alanine	A	Ala	$-CH_3$	X	-	-	-	X	X	-	67
Cysteine	C	Cys	$-CH_2SH$	-	8.18	-	acidic	X	X	-	86
Aspartic acid	D	Asp	$-CH_2COOH$	-	3.90	X	acidic	X	-	-	91
Glutamic acid	E	Glu	$-CH_2CH_2COOH$	-	4.07	X	acidic	-	-	-	109
Phenylalanine	F	Phe	$-CH_2C_6H_5$	X	-	-	-	-	-	Aromatic	135
Glycine	G	Gly	-H	X	-	-	-	X	X	-	48
Histidine	H	His	$-CH_2-C_3H_3N_2$	-	6.04	X	weak basic	-	-	Aromatic	118
Isoleucine	I	Ile	$-CH(CH_3)CH_2CH_3$	X	-	-	-	-	-	Aliphatic	124
Lysine	K	Lys	$-(CH_2)_4NH_2$	-	10.54	X	basic	-	-	-	135
Leucine	L	Leu	$-CH_2CH(CH_3)_2$	X	-	-	-	-	-	Aliphatic	124
Methionine	M	Met	$-CH_2CH_2SCH_3$	X	-	-	-	-	-	-	124
Asparagine	N	Asn	$-CH_2CONH_2$	-	-	X	-	X	-	-	96
Pyrrolysine	O	Pyl	$-(CH_2)_4NHCOC_4H_5NCH_3$	-	-	X	weak basic	-	-	-	
Proline	P	Pro	$-CH_2CH_2CH_2-$	X	-	-	-	X	-	-	90
Glutamine	Q	Gln	$-CH_2CH_2CONH_2$	-	-	X	weak basic	-	-	-	114
Arginine	R	Arg	$-(CH_2)_3NH-C(NH)NH_2$	-	12.48	X	strongly basic	-	-	-	148
Serine	S	Ser	$-CH_2OH$	-	5.68	X	weak acidic	X	X	-	73
Threonine	T	Thr	$-CH(OH)CH_3$	-	5.53	X	weak acidic	X	-	-	93
Selenocysteine	U	Sec	$-CH_2SeH$	-	5.73	-	acidic	X	X	-	
Valine	V	Val	$-CH(CH_3)_2$	X	-	-	-	X	-	Aliphatic	105
Tryptophan	W	Trp	$-CH_2C_8H_6N$	-	5.885	X	weak basic	-	-	Aromatic	163
Tyrosine	Y	Tyr	$-CH_2-C_6H_4OH$	-	10.46	X	weak acidic	-	-	Aromatic	141

Figure 2.2 - Amino acid side chain properties Wikipedia

- Glycine is nonessential to humans.
- Isoleucine (Ile/I) is an essential amino acid. It has a hydrophobic side chain and is also chiral in its side chain.
- Leucine (Leu/L) is a branched-chain amino acid that is hydrophobic and essential. Leucine is the only dietary amino acid reported to directly stimulate protein synthesis in muscle, but caution is in order, as 1) there are conflicting studies and 2) leucine toxicity is dangerous, resulting in "the four D's": diarrhea, dermatitis, dementia and death.
- Methionine (Met/M) is an essential amino acid that is one of two sulfur-containing amino acids - cysteine is the other. Methionine is non-polar and is the “initiator” amino acid in protein synthesis, being the first one incorporated into protein chains. In prokaryotic cells, the first methionine in a protein is formulated, i.e. chemically modified to incorporate a formyl functional group.

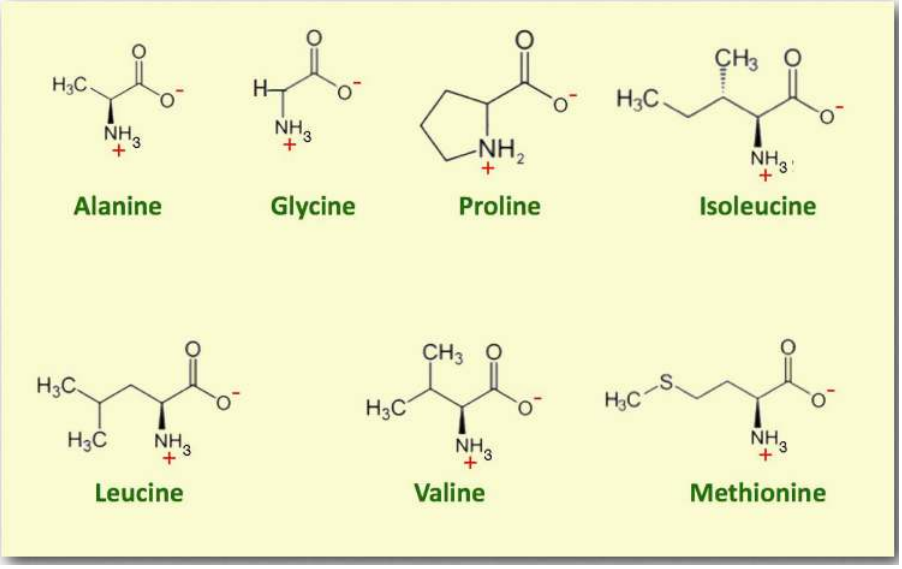


Figure 2.3 - Non-polar amino acids

- Proline (Pro/P) is the only amino acid found in proteins with an R-group that joins with its own α-amino group, making a secondary amine and a ring. Proline is a non-essential amino acid. It is the least flexible of the protein amino acids and thus gives conformational rigidity when present in a protein. Proline's presence in a protein affects its secondary structure. It is a disrupter of α-helices and β-strands. Proline is often hydroxylated in collagen (the reaction requires Vitamin C - ascorbate) and this has the effect of increasing the protein's conformational stability. Proline hydroxylation of hypoxia-inducible factor (HIF) serves as a sensor of oxygen levels and targets HIF for destruction when oxygen is plentiful.
- Valine (Val/V) is an essential, non-polar amino acid synthesized in plants. It is noteworthy in hemoglobin, for when it replaces glutamic acid at position number six, it causes hemoglobin to aggregate abnormally under low oxygen conditions, resulting in sickle cell disease.

Carboxyl Amino Acids

- Aspartic acid (Asp/D) is a non-essential amino acid with a carboxyl group in its R-group. It is readily produced by transamination of oxaloacetate. With a pKa of 3.9, aspartic acid's side chain is negatively charged at physiological pH.
- Glutamic acid (Glu/E), is a non-essential amino acid readily made by transamination of α- ketoglutarate. It is a neurotransmitter and has an R-group with a carboxyl group that readily ionizes (pKa = 4.1) at physiological pH. You may have seen some foods labeled as containing monosodium glutamate (MSG), which enhances savory umami taste of foods.

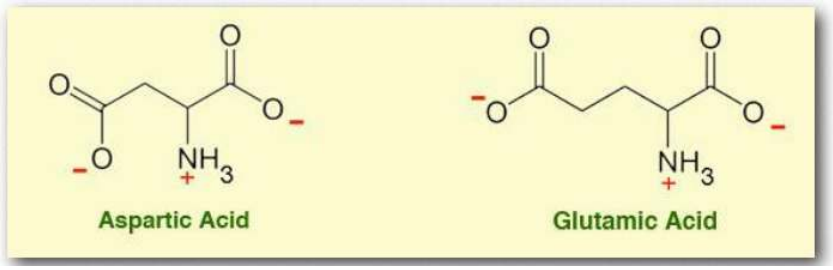


Figure 2.4 - Carboxyl amino acids

Amine-containing amino acids

- Arginine (Arg/R) is an amino acid that is, in some cases, essential, but non-essential in others. Premature infants cannot synthesize arginine. In addition, surgical trauma, sepsis, and burns increase demand for arginine. Most people, however, do not need arginine supplements. Arginine's side chain contains a complex guanidinium group with a pKa of over 12, making it positively charged at cellular pH.
- Histidine (His/H) is the only one of the proteinaceous amino acids to contain an imidazole functional group. It is an essential amino acid in humans and other mammals. With a side chain pKa of 6, it can easily have its charge changed by a slight change

in pH. Protonation of the ring results in two NH structures which can be drawn as two equally important resonant structures.



Figure 2.5 - Amine amino acids

- Lysine (Lys/K) is an essential amino acid encoded by AAA and AAG. It has an R-group that can readily ionize with a charge of +1 at physiological pH and can be posttranslationally modified to form acetyllysine, hydroxylysine, and methyllysine. There are many other possible post-translational modifications of lysine: it can also be ubiquitinated, sumoylated, neddylated, biotinylated, carboxylated, and pupylated (you do not have to memorize all these terms - just do not fear them if you encounter them in your study of biochemistry). Lysine is often added to animal feed because it is a limiting amino acid and is necessary for optimizing the growth of pigs and chickens.

Aromatic amino acids

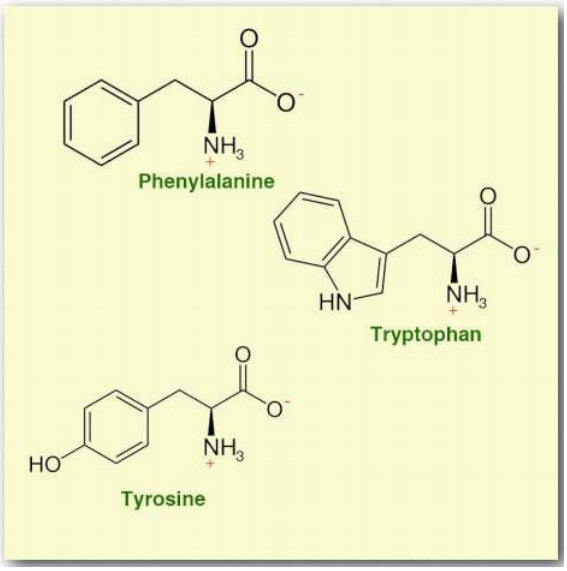


Figure 2.6 - Aromatic amino acids

- Phenylalanine (Phe/ F) is a non-polar, essential amino acid. It is a metabolic precursor of tyrosine. Inability to metabolize phenylalanine arises from the genetic disorder known as phenylketonuria. Phenylalanine is a component of the aspartame artificial sweetener.
- Tryptophan (Trp/W) is an essential amino acid containing an indole functional group. It is a metabolic precursor of serotonin, niacin, and (in plants) the auxin phytohormone. Though reputed to serve as a sleep aid, there are no clear research results indicating this.
- Tyrosine (Tyr/Y) is a non-essential amino acid. It is a target for phosphorylation in proteins by tyrosine protein kinases and plays a role in signaling processes. In dopaminergic cells of the brain, tyrosine hydroxylase converts tyrosine to l-dopa, an immediate precursor of dopamine. Dopamine, in turn, is a precursor of norepinephrine and epinephrine. Tyrosine is also a precursor of thyroid hormones and melanin.

Hydroxyl amino acids

- Serine (Ser/S) is one of three amino acids having an R-group with a hydroxyl in it (threonine and tyrosine are the others). Being able to hydrogen bond with water, it is classified as a polar amino acid. It is not essential for humans. Serine is precursor of

many important cellular compounds, including purines, pyrimidines, sphingolipids, folate, and of the amino acids glycine, cysteine, and tryptophan. The hydroxyl group of serine in proteins is a target for phosphorylation by certain protein kinases. Serine is also a part of the catalytic triad of serine proteases.

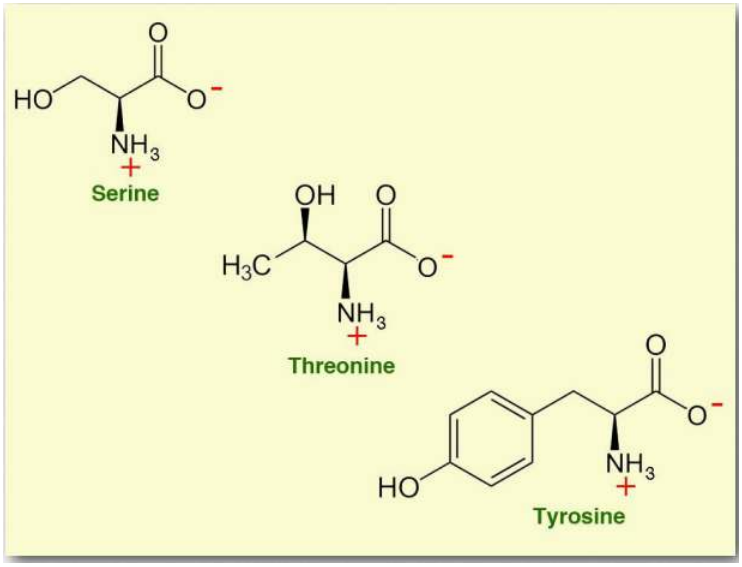


Figure 2.7 - Hydroxyl amino acids

- Threonine (Thr/T) is a polar amino acid that is essential. It is one of three amino acids bearing a hydroxyl group (serine and tyrosine are the others) and, as such, is a target for phosphorylation in proteins. It is also a target for O-glycosylation of proteins. Threonine proteases use the hydroxyl group of the amino acid in their catalysis and it is a precursor in one biosynthetic pathway for making glycine. In some applications, it is used as a pro-drug to increase brain glycine levels.

Tyrosine - see HERE.

Amino acid	Short	Abbrev.	Avg. mass (Da)	pI	pK_1 (α-COOH)	pK_2 (α-$^+NH_3$)
Alanine	A	Ala	89.09404	6.01	2.35	9.87
Cysteine	C	Cys	121.15404	5.05	1.92	10.70
Aspartic acid	D	Asp	133.10384	2.85	1.99	9.90
Glutamic acid	E	Glu	147.13074	3.15	2.10	9.47
Phenylalanine	F	Phe	165.19184	5.49	2.20	9.31
Glycine	G	Gly	75.06714	6.06	2.35	9.78
Histidine	H	His	155.15634	7.60	1.80	9.33
Isoleucine	I	Ile	131.17464	6.05	2.32	9.76
Lysine	K	Lys	146.18934	9.60	2.16	9.06
Leucine	L	Leu	131.17464	6.01	2.33	9.74
Methionine	M	Met	149.20784	5.74	2.13	9.28
Asparagine	N	Asn	132.11904	5.41	2.14	8.72
Pyrrolysine	O	Pyl	255.31			
Proline	P	Pro	115.13194	6.30	1.95	10.64
Glutamine	Q	Gln	146.14594	5.65	2.17	9.13
Arginine	R	Arg	174.20274	10.76	1.82	8.99
Serine	S	Ser	105.09344	5.68	2.19	9.21
Threonine	T	Thr	119.12034	5.60	2.09	9.10
Selenocysteine	U	Sec	168.053	5.47		
Valine	V	Val	117.14784	6.00	2.39	9.74
Tryptophan	W	Trp	204.22844	5.89	2.46	9.41
Tyrosine	Y	Tyr	181.19124	5.64	2.20	9.21

Figure 2.8 - Amino acid properties Wikipedia

Other amino acids

- Asparagine (Asn/N) is a non-essential amino acid. Its carboxyamide in the R-group gives it polarity. Asparagine is implicated in formation of acrylamide in foods cooked at high temperatures (deep frying) when it reacts with carbonyl groups. Asparagine can be made in the body from aspartate by an amidation reaction with an amine from glutamine. Breakdown of asparagine produces malate, which can be oxidized in the citric acid cycle.
- Cysteine (Cys/C) is the only amino acid with a sulfhydryl group in its side chain. It is nonessential for most humans, but may be essential in infants, the elderly and individuals who suffer from certain metabolic diseases. Cysteine's sulfhydryl group is readily oxidized to a disulfide when reacted with another one. In addition to being found in proteins, cysteine is also a component of the tripeptide, glutathione.

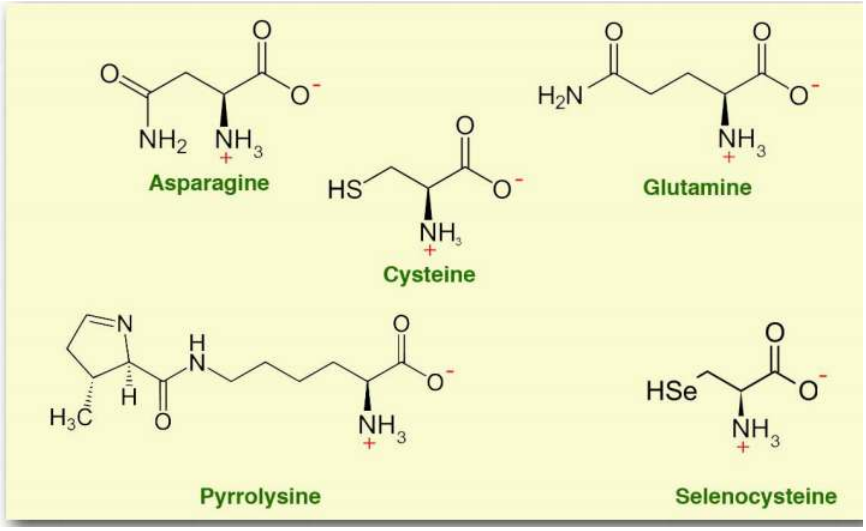


Figure 2.9 - Other amino acids

- Glutamine (Gln/Q) is an amino acid that is not normally essential in humans, but may be in individuals undergoing intensive athletic training or with gastrointestinal disorders. It has a carboxyamide side chain which does not normally ionize under physiological pHs, but which gives polarity to the side chain. Glutamine is coded for by CAA and CAG and is readily made by amidation of glutamate. Glutamine is the most abundant amino acid in circulating blood and is one of only a few amino acids that can cross the blood-brain barrier.
- Selenocysteine (Sec/U) is a component of selenoproteins found in all kingdoms of life. It is a component in several enzymes, including glutathione peroxidases and thioredoxin reductases. Selenocysteine is incorporated into proteins in an unusual scheme involving the stop codon UGA. Cells grown in the absence of selenium terminate protein synthesis at UGAs. However, when selenium is present, certain mRNAs which contain a selenocysteine insertion sequence (SECIS), insert selenocysteine when UGA is encountered. The SECIS element has characteristic nucleotide sequences and secondary structure base-pairing patterns. Twenty five human proteins contain selenocysteine.
- Pyrrolysine (Pyl/O) is a twenty second amino acid, but is rarely found in proteins. Like selenocysteine, it is not coded for in the genetic code and must be incorporated by unusual means. This occurs at UAG stop codons. Pyrrolysine is found in methanogenic archaean organisms and at least one methane-producing bacterium. Pyrrolysine is a component of methane-producing enzymes.

Ionizable groups

pKa values for amino acid side chains are very dependent upon the chemical environment in which they are present. For example, the R-group carboxyl found in aspartic acid has a pKa value of 3.9 when free in solution, but can be as high as 14 when in certain environments inside of proteins, though that is unusual and extreme. Each amino acid has at least one ionizable amine group (α-amine) and one ionizable carboxyl group (α- carboxyl). When these are bound in a peptide bond, they no longer ionize. Some, but not all amino acids have R-groups that can ionize. The charge of a protein then arises from the charges of the α-amine group, the α-carboxyl group. and the sum of the charges of the ionized R-groups. Titration/ionization of aspartic acid is depicted in Figure 2.10.

Ionization (or deionization) within a protein's structure can have a significant effect on the overall conformation of the protein and, since structure is related to function, a major impact on the activity of a protein.

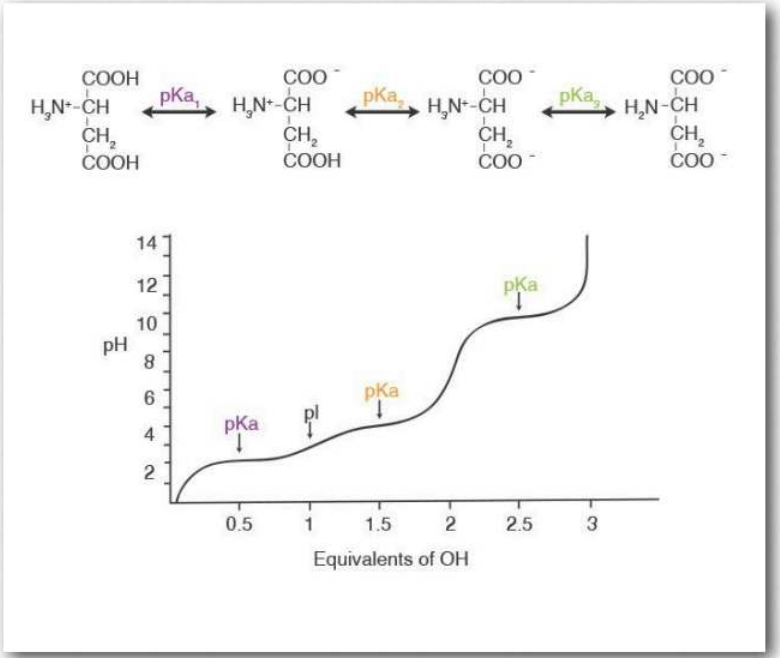


Figure 2.10 - Titration curve for aspartic acid Image by Penelope Irving

Most proteins have relatively narrow ranges of optimal activity that typically correspond to the environments in which they are found (Figure 2.11). It is worth noting that formation of peptide bonds between amino acids removes ionizable hydrogens from both the α- amine and α- carboxyl groups of amino acids. Thus, ionization/ deionization in a protein arises only from 1) the amino terminus; 2) carboxyl terminus; 3) R-groups; or 4) other functional groups (such as sulfates or phosphates) added to amino acids post-translationally - see below.

Carnitine

Not all amino acids in a cell are found in proteins. The most common examples include ornithine (arginine metabolism), citrulline (urea cycle), and carnitine (Figure 2.12). When fatty acids destined for oxidation are moved into the mitochondrion for that purpose, they travel across the inner membrane attached to carnitine. Of the two stereoisomeric forms, the L form is the active one. The molecule is synthesized in the liver from lysine and methionine.

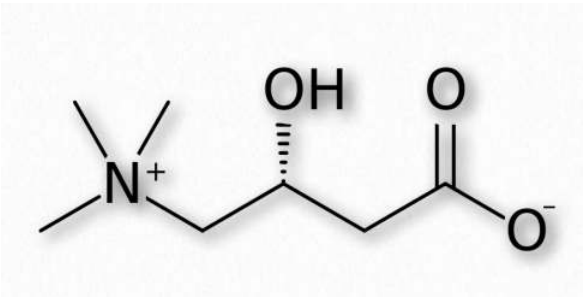


Figure 2.12 - L-Carnitine

From exogenous sources, fatty acids must be activated upon entry into the cytoplasm by being joined to coenzyme A. The CoA portion of the molecule is replaced by carnitine in the intermembrane space of the mitochondrion in a reaction catalyzed by carnitine acyltransferase I. The resulting acylcarnitine molecule is transferred across the inner mitochondrial membrane by the carnitineacylcarnitine translocase and then in the matrix of the mitochondrion, carnitine acyltransferase II replaces the carnitine with coenzyme A (Figure 6.88).

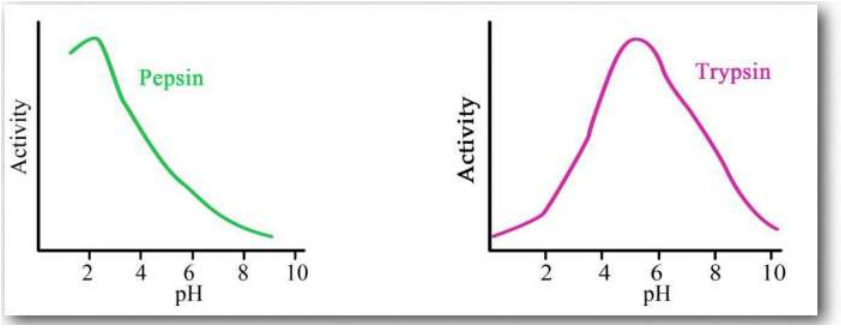


Figure 2.11 - Enzyme activity changes as pH changes Image by Aleia Kim

Catabolism of amino acids

We categorize amino acids as essential or non-essential based on whether or not an organism can synthesize them. All of the amino acids, however, can be broken down by all organisms. They are, in fact, a source of energy for cells, particularly during times of starvation or for people on diets containing very low amounts of carbohydrate. From a perspective of breakdown (catabolism), amino acids are categorized as glucogenic if they produce intermediates that can be made into glucose or ketogenic if their intermediates are made into acetyl-CoA. Figure 2.13 shows the metabolic fates of catabolism of each of the amino acids. Note that some amino acids are both glucogenic and ketogenic.

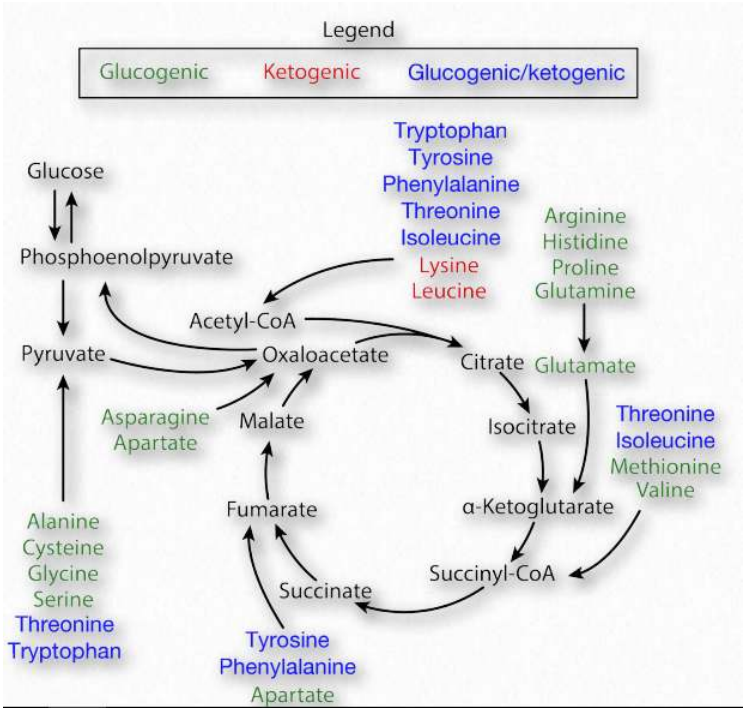


Figure 2.13 - Catabolism of amino acids. Some have more than one path. Image by Pehr Jacobson

Post-translational modifications

After a protein is synthesized, amino acid side chains within it can be chemically modified, giving rise to more diversity of structure and function (Figure 2.14). Common alterations include phosphorylation of hydroxyl groups of serine, threonine, or tyrosine. Lysine, proline, and histidine can have hydroxyls added to amines in their R-groups. Other modifications to amino acid residues in proteins include the addition of fatty acids (myristic acid or palmitic acid), isoprenoid groups, acetyl groups, methyl groups, iodine, carboxyl groups, or sulfates. These can have the effects of ionization (addition of phosphates/sulfates), deionization (addition of acetyl group to the R-group amine of lysine), or have no effect on charge at all. In addition, N-linked- and O-linked glycoproteins have carbohydrates covalently attached to side chains of asparagine and threonine or serine, respectively.

Some amino acids are precursors of important compounds in the body. Examples include epinephrine, thyroid hormones, L-dopa, and dopamine (all from tyrosine), serotonin (from tryptophan), and histamine (from histidine).

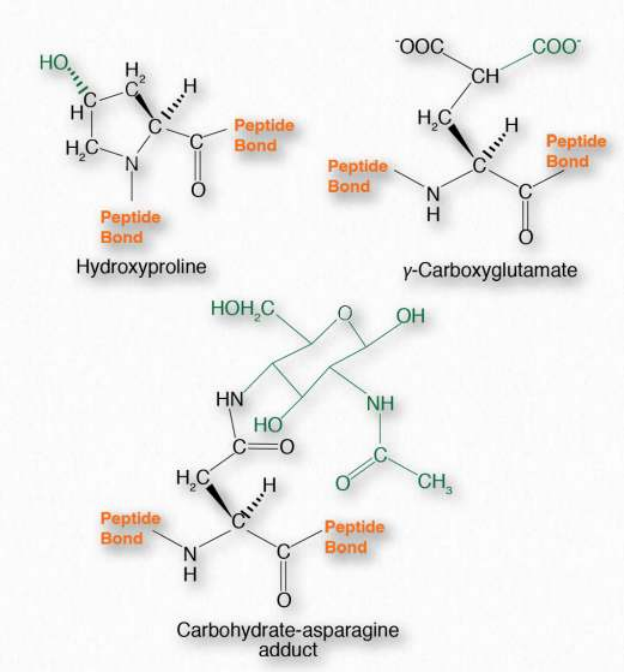


Figure 2.14 - Post-translationally modified amino acids. Modifications shown in green. Image by Penelope Irving

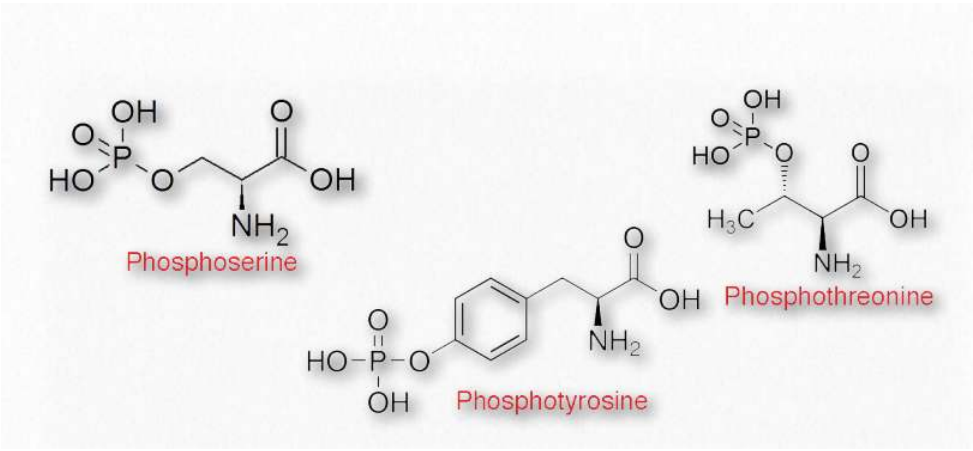


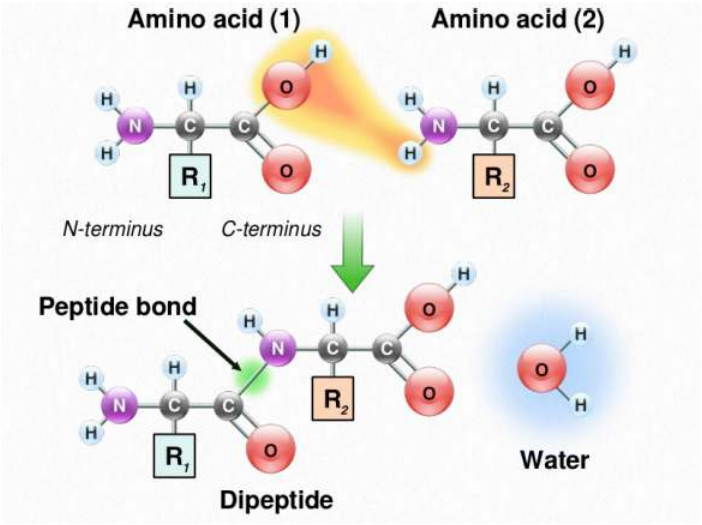
Figure 2.15 - Phosphorylated amino acids

Building Polypeptides

Although amino acids serve other functions in cells, their most important role is as constituents of proteins. Proteins, as we noted earlier, are polymers of amino acids.

Amino acids are linked to each other by peptide bonds, in which the carboxyl group of one amino acid is joined to the amino group of the next, with the loss of a molecule of water. What's left of an amino acid after it has been incorporated into a protein is called an amino acid **residue**. A chain made up of just a few amino acid residues is called an oligopeptide (oligo=few) while a typical protein, which is made up of many amino acids is called a polypeptide (poly=many). The end of the peptide that has a free amino group is called the N-terminus (it has NH3+ group), while the end with the free carboxyl is termed the C-terminus (C for carboxyl).

A.



B.

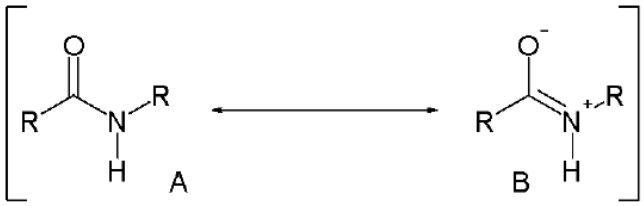


Figure 2.16 A. Formation of a peptide bond B. Amide Resonance structures (by https://commons.wikimedia.org/wiki/F...eResonance.png)

Note that peptide bond, an amide bond, is strong and does not have free rotation around it because of the partial double bond character (see resonance structure). Resonance structures also illustrate why nitrogen of the amino acid residue (that used to be an ionizable amino group in the free amino acid) can no longer be protonated. Carboxyl group of the free amino acid gives rise to the carbonyl group of the amino acid residue and also cannot be ionized. So if you have two free amino acids in solution: Ala and Asp, they will have 5 pKa values; once they are joined by peptide bond into dipeptide Ala-Asp, they will only have three pKas: amino group pKa of Ala, carboxyl group pKa of Asp and side-chain group pKa of Asp. It's important to note that by default, amino acid sequence of a peptide is always written from N- to C-terminus. So Ala-Asp dipeptide has alanine at the N-terminus, while Asp-Ala is a different dipeptide that has Aspartic acid at the N-terminus and alanine at the C-terminus.

As we've noted before, the function is dependent on structure, and the string of amino acids must fold into a specific 3-D shape, or conformation, in order to make a functional protein. The folding of polypeptides into their functional forms is the topic of the next section.

2.1: Structure and Function - Amino Acids is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by LibreTexts.

- **2.2: Structure & Function - Amino Acids** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

2.2: Structure and Function- Proteins I

Source: BiochemFFA_2_2.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

Proteins are the workhorses of the cell. Virtually everything that goes on inside of cells happens as a result of the actions of proteins. Among other things, protein enzymes catalyze the vast majority of cellular reactions, mediate signaling, give structure both to cells and to multicellular organisms, and exert control over the expression of genes. Life, as we know it, would not exist if there were no proteins. The versatility of proteins arises because of their varied structures.

Proteins are made by linking together amino acids, with each protein having a characteristic and unique amino acid sequence. To get a sense for the diversity of proteins that can be made using 20 different amino acids, consider that the number of different combinations possible with 20 amino acids is 20^n, where n=the number of amino acids in the chain. It becomes apparent that even a dipeptide made of just two amino acids joined together gives us 20^2 = 400 different combinations. If we do the calculation for a short peptide of 10 amino acids, we arrive at an enormous 10,240,000,000,000 combinations. Most proteins are much larger than this, making the possible number of proteins with unique amino acid sequences unimaginably huge.

Levels of Structure

The significance of the unique sequence, or order, of amino acids, known as the protein's primary structure, is that it dictates the 3-D conformation the folded protein will have. This conformation, in turn, will determine the function of the protein. We shall examine protein structure at four distinct levels (Figure 2.17) - 1) how sequence of the amino acids in a protein (primary structure) gives identity and characteristics to a protein (Figure 2.18); 2) how local interactions between one part of the polypeptide backbone and another affect protein shape (secondary structure); 3) how the polypeptide chain of a protein can fold to allow amino acids to interact with each other that are not close in primary structure (tertiary structure); and 4) how different polypeptide chains interact with each other within a multi-subunit protein (quaternary structure).

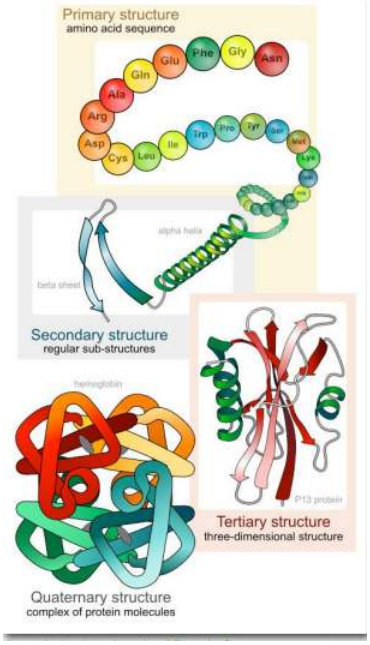


Figure 2.17 - Four Levels of Protein Structure

At this point, we should provide a couple of definitions. We use the term polypeptide to refer to a single polymer of amino acids. It may or may not have folded into its final, functional form. The term protein is sometimes used interchangeably with polypeptide, as in "protein synthesis". It is generally used, however, to refer to a folded, functional molecule that may have one or more subunits (made up of individual polypeptides). Thus, when we use the term protein, we are usually referring to a functional, folded polypeptide or peptides. Structure is essential for function. If you alter the structure, you alter the function - usually, but not always, this means you lose all function. For many proteins, it is not difficult to alter the structure.

Proteins are flexible, not rigidly fixed in structure. As we shall see, it is the flexibility of proteins that allows them to be amazing catalysts and allows them to adapt to, respond to, and pass on signals upon binding of other molecules or proteins. However, proteins are not infinitely flexible. There are constraints on the conformations that proteins can adopt and these constraints govern the conformations that proteins display.

Subtle changes

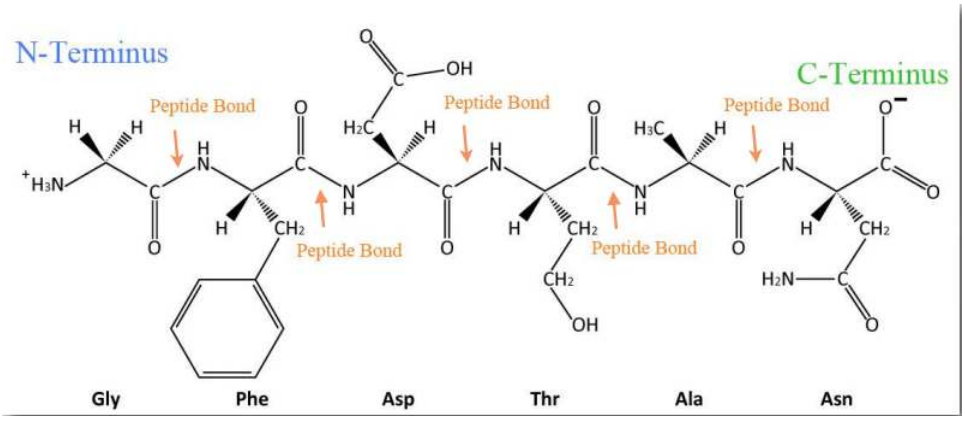


Figure 2.18 - Sequence of a simple polypeptide Wikipedia

Even very tiny, subtle changes in protein structure can give rise to big changes in the behavior of proteins. Hemoglobin, for example, undergoes an incredibly small structural change upon binding of one oxygen molecule, and that simple change causes the remainder of the protein to gain a considerably greater affinity for oxygen that the protein didn't have before the structural change.

Sequence, structure and function

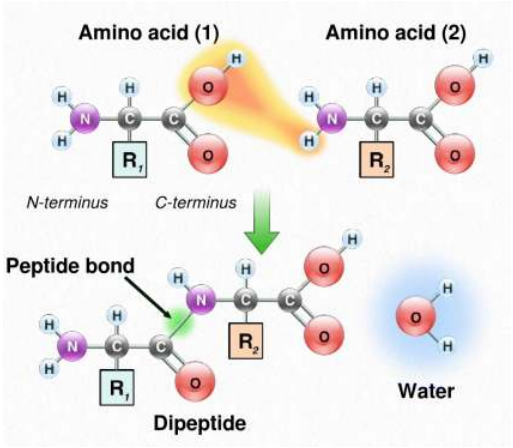


Figure 2.19 Linking of amino acids through peptide bond formation

As discussed earlier, the number of different amino acid sequences possible, even for short peptides, is very large. No two proteins with different amino acid sequences (primary structure) have identical overall structure. The unique amino acid sequence of a protein is reflected in its unique folded structure. This structure, in turn, determines the protein's function. This is why mutations that alter amino acid sequence can affect the function of a protein.

Protein Synthesis

Synthesis of proteins occurs in the ribosomes and proceeds by joining the carboxyl terminus of the first amino acid to the amino terminus of the next one (Figure 2.19). The end of the protein that has the free α-amino group is referred to as the amino terminus or N-terminus. The other end is called the carboxyl terminus or C-terminus , since it contains the only free α-carboxyl group. All of the other α-amino groups and α-carboxyl groups are tied up in forming peptide Figure 2.19 Linking of amino acids through peptide bond formation bonds that join adjacent amino acids together. Proteins are synthesized starting with the amino terminus and ending at the carboxyl terminus.

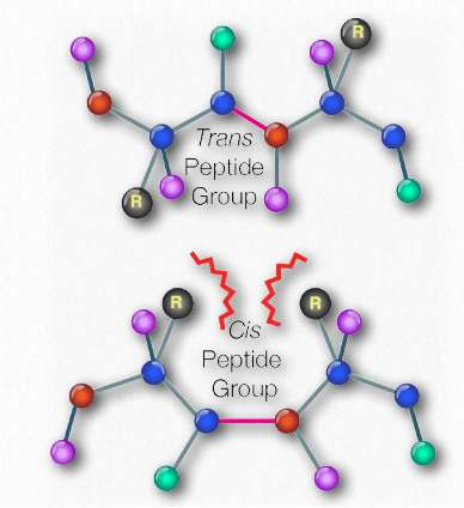


Figure 2.20 - Cis vs trans orientation of R-groups around peptide bond Image by Aleia Kim

Schematically, in Figure 2.18, we can see how sequential R-groups of a protein are arranged in an alternating orientation on either side of the polypeptide chain. Organization of R-groups in this fashion is not random. Steric hindrance can occur when consecutive R-groups are oriented on the same side of a peptide backbone (Figure 2.20)

Primary Structure

Primary structure is the ultimate determinant of the overall conformation of a protein. The primary structure of any protein arrived at its current state as a result of mutation and selection over evolutionary time. Primary structure of proteins is mandated by the sequence of DNA coding for it in the genome. Regions of DNA specifying proteins are known as coding regions (or genes).

The base sequences of these regions directly specify the sequence of amino acids in proteins, with a one-to-one correspondence between the codons (groups of three consecutive bases) in the DNA and the amino acids in the encoded protein. The sequence of codons in DNA, copied into messenger RNA, specifies a sequence of amino acids in a protein. (Figure 2.21).

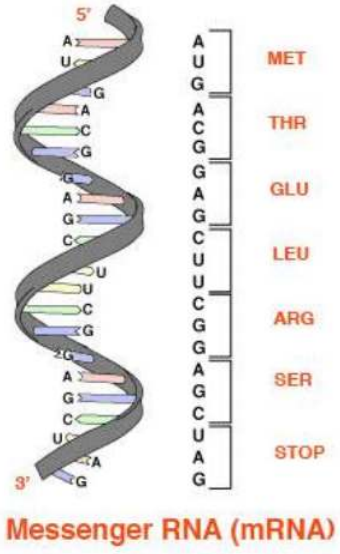


Figure 2.21 - From RNA to amino acids - the genetic code Wikipedia

The order in which the amino acids are joined together in protein synthesis starts defining a set of interactions between amino acids even as the synthesis is occurring. That is, a polypeptide can fold even as it is being made. The order of the R-group structures and resulting interactions are very important because early interactions affect later interactions. This is because interactions start establishing structures - secondary and tertiary. If a helical structure (secondary structure), for example, starts to form, the

possibilities for interaction of a particular amino acid Rgroup may be different than if the helix had not formed (Figure 2.22). R-group interactions can also cause bends in a polypeptide sequence (tertiary structure) and these bends can create (in some cases) opportunities for interactions that wouldn't have been possible without the bend or prevent (in other cases) similar interaction possibilities.

Secondary Structure

As protein synthesis progresses, interactions between amino acids close to each other begin to occur, giving rise to local patterns called secondary structure. These secondary structures include the well known α- helix and β-strands. Both were predicted by Linus Pauling, Robert Corey, and Herman Branson in 1951. Each structure has unique features.

α-helix

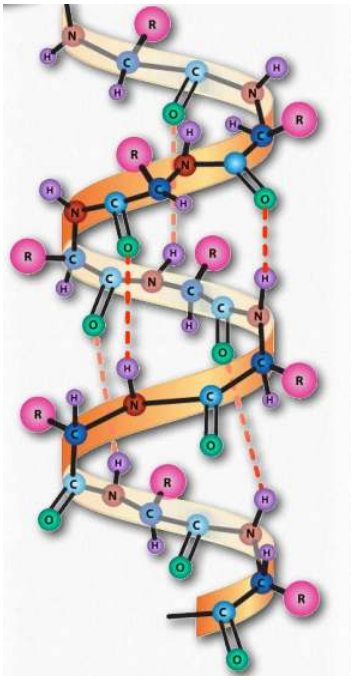


Figure 2.22 - The α-helix. Hydrogen bonds (dotted lines) between the carbonyl oxygen and the amine hydrogen stabilize the structure. Image by Aleia Kim

The α-helix has a coiled structure, with 3.6 amino acids per turn of the helix (5 helical turns = 18 amino acids). Helices are predominantly right handed - only in rare cases, such as in sequences with many glycines can left handed α- helices form. In the α-helix, hydrogen bonds form between C=O groups and N-H groups in the polypeptide backbone that are four amino acids distant. These hydrogen bonds are the primary forces stabilizing the α-helix.

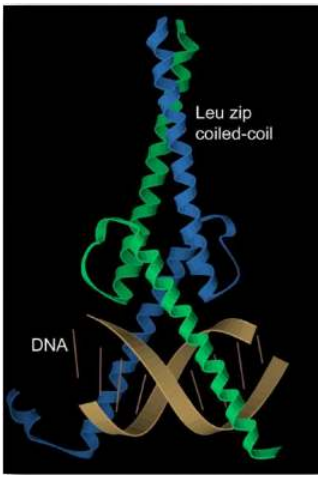


Figure 2.23 - α-helices in a protein with a leucine zipper structural domain. The α-helices are shown in blue and green and are bound to a DNA double helix in brown.

We use the terms rise, repeat, and pitch to describe the parameters of any helix. The repeat is the number of residues in a helix before it begins to repeat itself. For an α-helix, the repeat is 3.6 amino acids per turn of the helix. The rise is the distance the helix elevates with addition of each residue. For an α-helix, this is 0.15 nm per amino acid. The pitch is the distance between complete turns of the helix. For an α-helix, this is 0.54 nm. The stability of an α-helix is enhanced by the presence of the amino acid aspartate.



Figure 2.24 - α-helix sculpture outside Linus Pauling's boyhood home Wikipedia

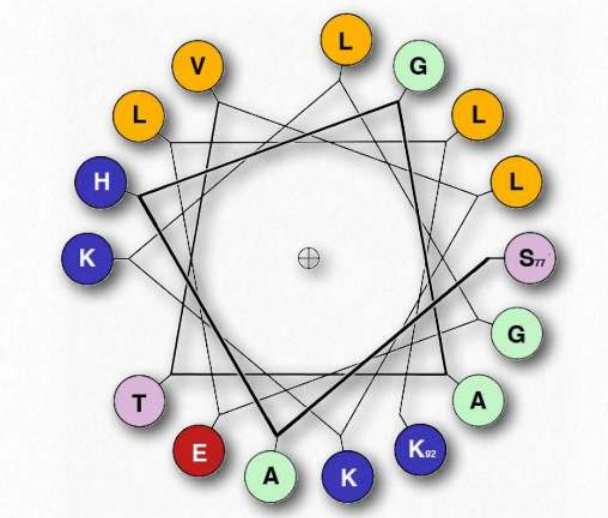


Figure 2.25 - Helical Wheel Representation of an α-Helix. The one letter genetic code is used. The helix starts at Serine #77 at the right and ends at lysine #92 in the lower right. Hydrophobic amino acids are shown in yellow and ionizing amino acids are shown in blue. Hydrophobic amino acids tend to interact with each other and not with ionizing amino acids. Wikipedia

β strand/sheet

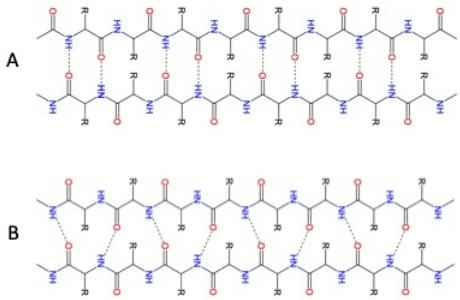


Figure 2.26 - β sheets: A. anti-parallel beta-sheet B parallel beta-sheet (from https://en.wikipedia.org/wiki/Beta_sheet)

In contrast to the compact coiled shape of the alpha-helix, beta-strands are more extended but contain bends that are sometimes referred to as pleats, like the pleats in a curtain. β-strands can be organized to form elaborately organized structures, such as sheets, barrels, and other arrangements.

β-sheets are stabilized by hydrogen bonds between carbonyl oxygen atoms and hydrogens of amine groups in the polypeptide backbone (Figure 2.28) from distant regions. In a higher order structure, strands can be arranged parallel (amino to carboxyl orientations the same) or anti-parallel (amino to carboxyl orientations opposite of each other (in Figure 2.27, the direction of the strand is shown by the arrowhead in the ribbon diagrams).

Turns

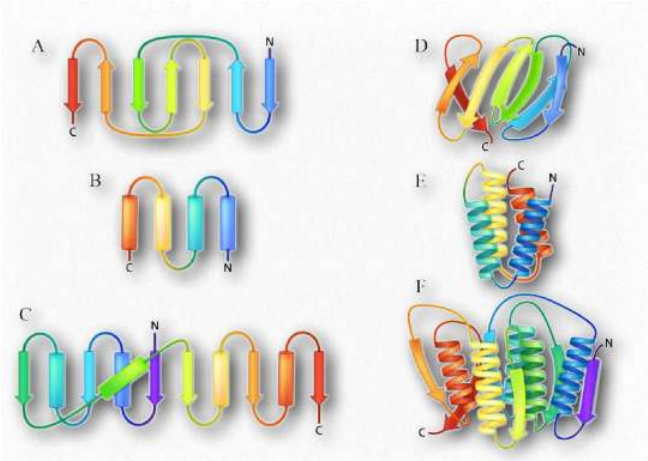


Figure 2.27 - Ribbon depictions of supersecondary β-sheets (A-D) and α-helix arrangements (E-F) Image by Aleia Kim

Turns (sometimes called reverse turns) are a type of secondary structure that, as the name suggests, causes a turn in the structure of a polypeptide chain. Turns give rise to tertiary structure ultimately, causing interruptions in the secondary structures (α- helices and β-strands) and often serve as connecting regions between two regions of secondary structure in a protein. Proline and glycine play common roles in turns, providing less flexibility (starting the turn) and greater flexibility (facilitating the turn), respectively.

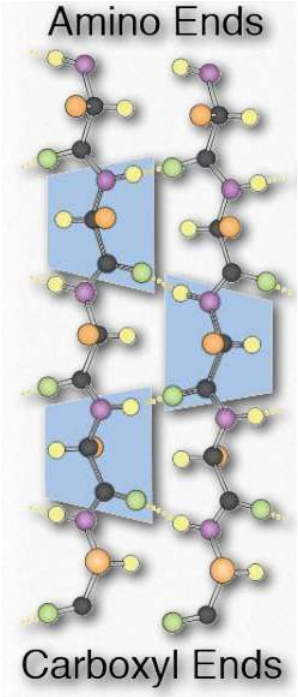


Figure 2.28 - Components of a β-sheet in a parallel arrangement. H-bonds in yellow. Image by Aleia Kim

There are at least five types of turns, with numerous variations of each giving rise to many different turns. The five types of turns are

• δ-turns - end amino acids are separated by one peptide bond

• γ-turns - separation by two peptide bonds

•β-turns - separation by three peptide bonds

•α-turns - separation by four peptide bonds

•π-turns - separation by five bonds

Of these, the β-turns are the most common form and the δ-turns are theoretical, but unlikely, due to steric limitations. Figure 2.29 depicts a β- turn.

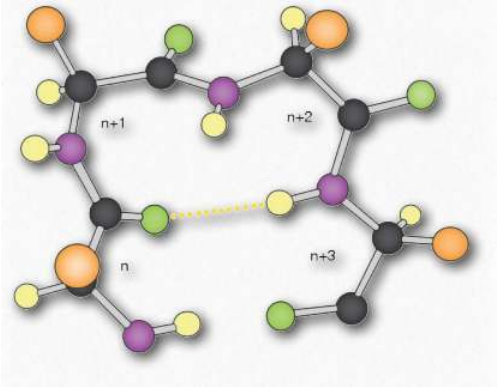


Figure 2.29 - β-turn. R-groups are shown in orange, hydrogens in yellow, carbons in charcoal, nitrogens in purple, and oxygens in green. A stabilizing hydrogen bond is indicated with the dotted line. Image by Aleia Kim

Notice that the arrangement of the backbone is limited by the partial double-bond character of the peptide bond: the backbone can exhibit free rotation around the bonds to the alpha-carbon of each amino acid residue, but not around the peptide bond itself.

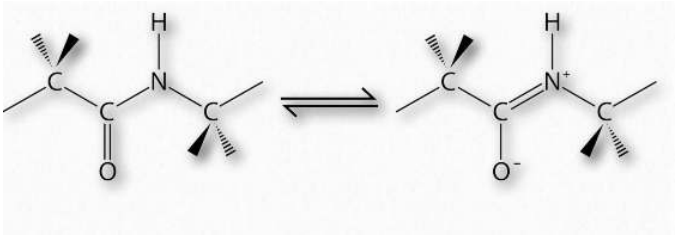


Figure 2.30 - Resonance of the peptide bond Wikipedia

Hydrophobicity

Amino Acid Hydropathy Scores

Amino Acid	One Letter Code	Hydropathy Score
Isoleucine	I	4.5
Valine	V	4.2
Leucine	L	3.8
Phenylalanine	F	2.8
Cysteine	C	2.5
Methionine	M	1.9
Alanine	A	1.8
Glycine	G	-0.4
Threonine	T	-0.7
Tryptophan	W	-0.9
Serine	S	-0.8
Tyrosine	Y	-1.3
Proline	P	-1.6
Histidine	H	-3.2
Glutamic acid	E	-3.5
Glutamine	Q	-3.5
Aspartic acid	D	-3.5
Asparagine	N	-3.5
Lysine	K	-3.9
Arginine	R	-4.5

Table 2.4 - Hydropathy Scores

The chemistry of amino acid R-groups affects the structures they are most commonly found in. Subsets of their chemical properties can give clues to structure and, sometimes, cellular location. A prime example is the hydrophobicity (water-avoiding tendencies) of some R-groups. Given the aqueous environment of the cell, such R-groups are not likely to be on the outside surface of a folded protein.

However, this rule does not hold for regions of protein that may be embedded within the lipid bilayers of cellular/ organelle membranes. This is because the region of such proteins that form the transmembrane domains are buried in the hydrophobic environment in the middle of the lipid bilayer.

Not surprisingly, scanning primary sequences for specifically sized/spaced stretches of hydrophobic amino acids can help to identify proteins found in membranes. Table 2.4 shows hydrophobicity values for R-groups of the amino acids. In this set, the scale runs from positive values (hydrophobic) to negative values (hydrophilic).

[Note from AG: several sections of text were removed - please, ignore non-consecutive numbering of the figures here...the figures 2.31-2.36 are removed intentionally]

Random coils

Some sections of a protein assume no regular, discernible structure and are sometimes said to lack secondary structure, though they may have hydrogen bonds. Such segments are described as being in random coils and may have fluidity to their structure that results in them having multiple stable forms. Random coils are identifiable with spectroscopic methods and nuclear magnetic resonance (NMR) in which distinctive signals are observed.



Figure 2.37 - Ribbon depiction of a β-hairpin. Shown are two β strands in turquoise interacting with each other.

Structural motifs = super-secondary structure

Another element of protein structure is harder to categorize because it incorporates elements of secondary and tertiary structure. Dubbed supersecondary structure (or structural motifs), these structures contain multiple nearby secondary structure components arranged in a specific way and that appear in multiple proteins. Since there are many ways of making secondary structures from different primary structures, so too can similar motifs arise from different primary sequences. An example of a structural motif is shown in Figure 2.37.

Tertiary structure

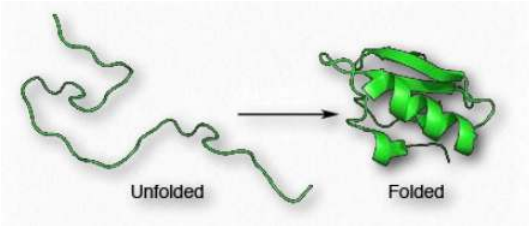


Figure 2.38 - Folding of a polypeptide chain

Proteins are distinguished from each other by the sequence of amino acids comprising them. The sequence of amino acids of a protein determines protein shape, since the chemical properties of each amino acid are forces that give rise to intermolecular

interactions to begin to create secondary structures, such as α-helices and β-strands. The sequence also defines turns and random coils that play important roles in the process of protein folding.

Since shape is essential for protein function, the sequence of amino acids gives rise to all of the properties a protein has. As protein synthesis proceeds, individual components of secondary structure start to interact with each other, giving rise to folds that bring amino acids close together that are not near each other in primary structure (Figure 2.38). At the tertiary level of structure, interactions among the R-groups of the amino acids in the protein, as well as between the polypeptide backbone and amino acid side groups play a role in folding.

Globular proteins

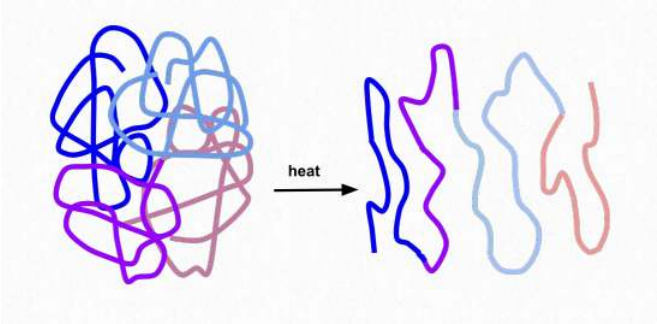


Figure 2.39 - Unfolding (denaturation) of a protein Wikipedia

Folding gives rise to distinct 3-D shapes in proteins that are non-fibrous. These proteins are called globular. A globular protein is stabilized by the same forces that drive its formation. These include ionic interactions, hydrogen bonding, hydrophobic forces, ionic bonds, disulfide bonds and metallic bonds. Treatments such as heat, pH changes, detergents, urea and mercaptoethanol overpower the stabilizing forces and cause a protein to unfold, losing its structure and (usually) its function (Figure 2.39). The ability of heat and detergents to denature proteins is why we cook our food and wash our hands before eating - such treatments denature the proteins in the microorganisms on our hands. Organisms that live in environments of high temperature (over 50°C) have proteins with changes in stabilizing forces - additional hydrogen bonds, additional salt bridges (ionic interactions), and compactness may all play roles in keeping these proteins from unfolding.

Protein stabilizing forces

Before considering the folding process, let us consider some of the forces that help to stabilize proteins.

Hydrogen bonds

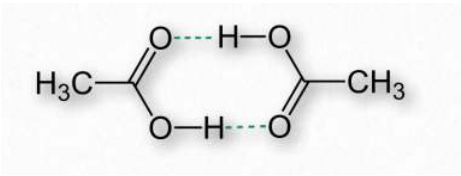


Figure 2.40 - Hydrogen bonds (dotted lines) between two molecules of acetic acid

Hydrogen bonds arise as a result of partially charged hydrogens found in covalent bonds. This occurs when the atom the hydrogen is bonded to has a greater electronegativity than hydrogen itself does, resulting in hydrogen having a partial positive charge because it is not able to hold electrons close to itself (Figure 2.40).

Hydrogen partially charged in this way is attracted to atoms, such as oxygen and nitrogen that have partial negative charges, due to having greater electronegativities and thus holding electrons closer to themselves. The partially positively charged hydrogens are called donors, whereas the partially negative atoms they are attracted to are called acceptors. (See Figure 1.30).

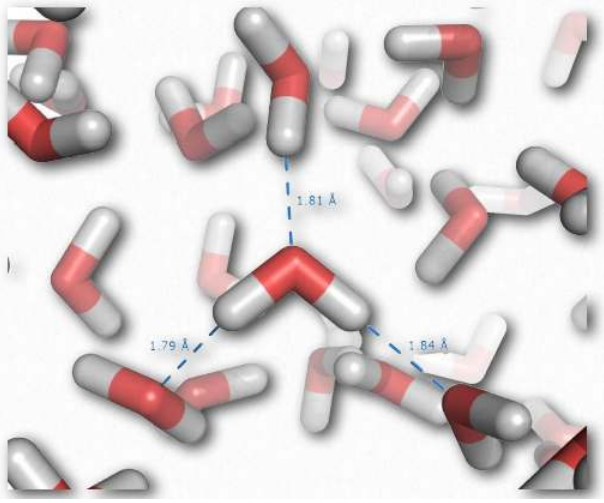


Figure 2.41 - Hydrogen bonding in liquid water Wikipedia

Individual hydrogen bonds are much weaker than a covalent bond, but collectively, they can exert strong forces. Consider liquid water, which contains enormous numbers of hydrogen bonds (Figure 2.41). These forces help water to remain liquid at room temperature. Other molecules lacking hydrogen bonds of equal or greater molecular weight than water, such as methane or carbon dioxide, are gases at the same temperature. Thus, the intermolecular interactions between water molecules help to “hold” water together and remain a liquid. Notably, only by raising the temperature of water to boiling are the forces of hydrogen bonding overcome, allowing water to become fully gaseous.

Hydrogen bonds are important forces in biopolymers that include DNA, proteins, and cellulose. All of these polymers lose their native structures upon boiling. Hydrogen bonds between amino acids that are close to each other in primary structure can give rise to regular repeating structures, such as helices or pleats, in proteins (secondary structure).

Ionic interactions

Ionic interactions are important forces stabilizing protein structure that arise from ionization of R-groups in the amino acids comprising a protein. These include the carboxyl amino acids (HERE), the amine amino acids as well as the sulfhydryl of cysteine and sometimes the hydroxyl of tyrosine.

Hydrophobic forces

Hydrophobic forces stabilize protein structure as a result of interactions that favor the exclusion of water. Non-polar amino acids (commonly found in the interior of proteins) favor associating with each other and this has the effect of excluding water. The excluded water has a higher entropy than water interacting with the hydrophobic side chains. This is because water aligns itself very regularly and in a distinct pattern when interacting with hydrophobic molecules.

When water is prevented from having these kinds of interactions, it is much more disordered that it would be if it could associate with the hydrophobic regions. It is partly for this reason that hydrophobic amino acids are found in protein interiors - so they can exclude water and increase entropy.

Disulfide bonds

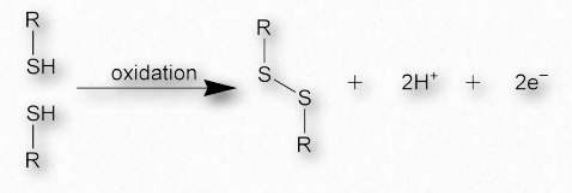


Figure 2.42 - Formation of a disulfide bond

Disulfide bonds, which are made when two sulfhydryl side-chains of cysteine are brought into close proximity, covalently join together different protein regions and can give great strength to the overall structure (Figures 2.42 & 2.43). An Ode to Protein Structure by Kevin Ahern The twenty wee amino A's Define a protein many ways Their order in a peptide chain Determines forms that proteins gain And when they coil, it leaves me merry Cuz that makes structures secondary It's tertiary, I am told That happens when a protein folds But folded chains are downright scary When put together quaternary They're nature's wonders, that's for sure Creating problems, making cures A fool can fashion peptide poems But proteins come from ribosoems These joined residues of cysteine are sometimes referred to as cystine. Disulfide bonds are the strongest of the forces stabilizing protein structure.

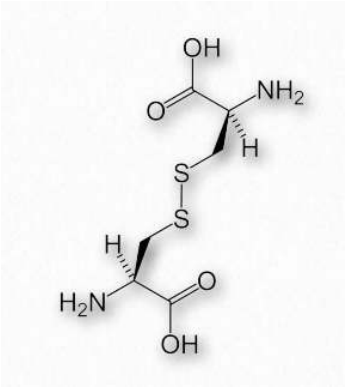


Figure 2.43 - Cystine - Two cysteines joined by a disulfide bond

van der Waals forces

van der Waals forces is a term used to describe various weak interactions, including those caused by attraction between a polar molecule and a transient dipole, or between two temporary dipoles. van der Waals forces are dynamic because of the fluctuating nature of the attraction, and are generally weak in comparison to covalent bonds, but can, over very short distances, be significant.

Post-translational modifications

Post-translational modifications can result in formation of covalent bonds stabilizing proteins as well. Hydroxylation of lysine and proline in strands of collagen can result in cross-linking of these groups and the resulting covalent bonds help to strengthen and stabilize the collagen.

Folding models

Two popular models of protein folding are currently under investigation. In the first (diffusion collision model), a nucleation event begins the process, followed by secondary structure formation. Collisions between the secondary structures (as in the β-hairpin in Figure 2.37) allow for folding to begin. By contrast, in the nucleation-condensation model, the secondary and tertiary structures form together.

Folding in proteins occurs fairly rapidly (0.1 to 1000 seconds) and can occur during synthesis - the amino terminus of a protein can start to fold before the carboxyl terminus is even made, though that is not always the case.

Folding process

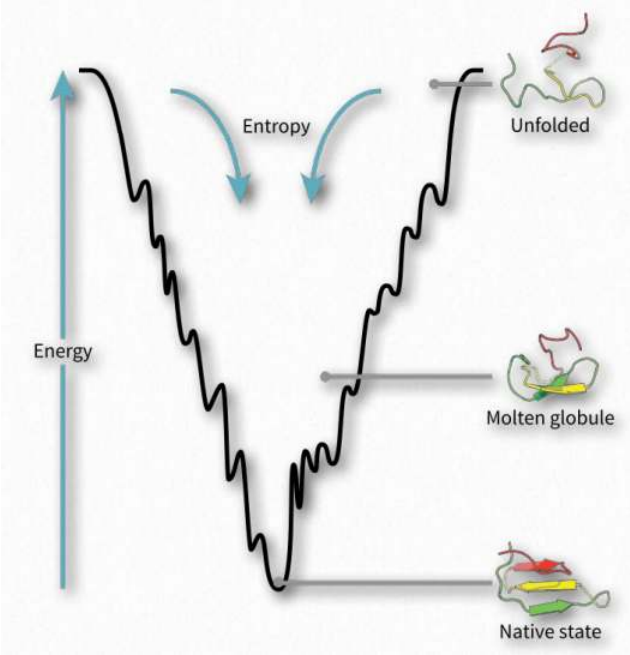


Figure 2.44 Folding funnel energy model of folding Wikipedia

Protein folding is hypothesized to occur in a “folding funnel” energy landscape in which a folded protein’s native state corresponds to the minimal free energy possible in conditions of the medium (usually aqueous solvent) in which the protein is dissolved. As seen in the diagram (Figure 2.44), the energy funnel has numerous local minima (dips) in which a folding protein can become trapped as it moves down the energy plot. Other factors, such as temperature, electric/magnetic fields, and spacial considerations likely play roles.

If external forces affect local energy minima during folding, the process and end-product can be influenced. As the speed of a car going down a road will affect the safety of the journey, so too do energy considerations influence and guide the folding process, resulting in fully functional, properly folded proteins in some cases and misfolded “mistakes” in others.

Getting stuck

As the folding process proceeds towards an energy minimum (bottom of the funnel in Figure 2.44), a protein can get “stuck” in any of the local minima and not reach the final folded state. Though the folded state is, in general, more organized and therefore has reduced entropy than the unfolded state, there are two forces that overcome the entropy decrease and drive the process forward.

The first is the magnitude of the decrease in energy as shown in the graph. Since $\Delta G = \Delta H - T\Delta S$, a decrease in ΔH can overcome a negative ΔS to make ΔG negative and push the folding process forward. Favorable (decreased) energy conditions arise with formation of ionic bonds, hydrogen bonds, disulfide bonds, and metallic bonds during the folding process. In addition, the hydrophobic effect increases entropy by allowing hydrophobic amino acids in the interior of a folded protein to exclude water, thus countering the impact of the ordering of the protein structure by making the ΔS less negative.

Spectroscopy

Because of our inability to accurately predict tertiary structure based on amino acid sequence, proteins structures are actually determined using techniques of spectroscopy. In these approaches, proteins are subjected to varied forms of electromagnetic radiation and the ways they interact with the radiation allows researchers to determine atomic coordinates at Angstrom resolution from electron densities (by X-ray crystallography) and how nuclei spins interact (by NMR).

Diseases of protein misfolding

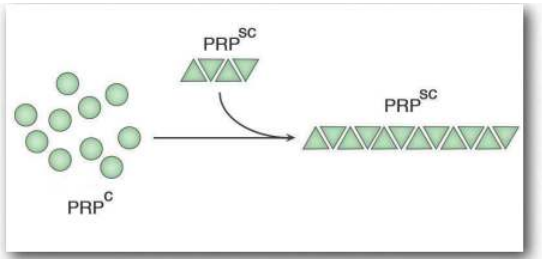


Figure 2.45 - Misfolding of the normal PRPc protein induced by PRPsc Image by Penelope Irving

The proper folding of proteins is essential to their function. It follows then that misfolding of proteins (also called proteopathy) might have consequences. In some cases, this might simply result in an inactive protein. Protein misfolding also plays a role in numerous diseases, such as Mad Cow Disease, Alzheimers, Parkinson's Disease, and CreutzfeldJakob disease. Many, but not all, misfolding diseases affect brain tissue.



Figure 2.46 - Cows with Mad Cow Disease lose their ability to stand

Insoluble deposits

Misfolded proteins will commonly form aggregates called amyloids that are harmful to tissues containing them because they change from being soluble to insoluble in water and form deposits. The process by which misfolding (Figure 2.45) occurs is not completely clear, but in many cases, it has been demonstrated that a "seed" protein which is misfolded can induce the same misfolding in other copies of the same protein. These seed proteins are known as prions and they act as infectious agents, resulting in the spread of disease. The list of human diseases linked to protein misfolding is long and continues to grow. A Wikipedia link is HERE.

Prions

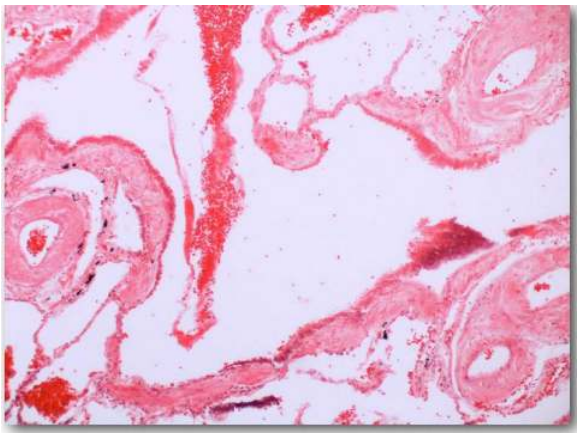


Figure 2.47 - Diffuse amyloidosis in a blood vessel (red dots) Wikipedia

Prions are infectious protein particles that cause transmissible spongiform encephalopathies (TSEs), the best known of which is Mad Cow disease. Other manifestations include the disease, scrapie, in sheep, and human diseases, such as CreutzfeldtJakob disease (CJD), Fatal Familial Insomnia, and kuru. The protein involved in these diseases is a membrane protein called PrP. PrP is encoded in the genome of many organisms and is found in most cells of the body. PrPc is the name given to the structure of PrP that is normal and not associated with disease. PrPSc is the name given to a misfolded form of the same protein, that is associated with the development of disease symptoms (Figure 2.45).

Misfolded

The misfolded PrPSc is associated with the TSE diseases and acts as an infectious particle. A third form of PrP, called PrPres can be found in TSEs, but is not infectious. The 'res' of PrPres indicates it is protease resistant. It is worth noting that all three forms of PrP have the same amino acid sequence and differ from each other only in the ways in which the polypeptide chains are folded. The most dangerously misfolded form of PrP is PrPSc, because of its ability to act like an infectious agent - a seed protein that can induce misfolding of PrPc , thus converting it into PrPSc.

Function

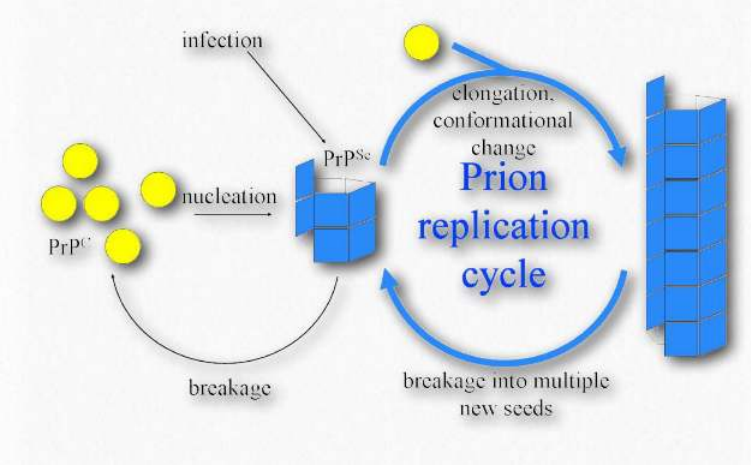


Figure 2.48 - One model of prion propagation Wikipedia

The function of PrPc is unknown. Mice lacking the PrP gene do not have major abnormalities. They do appear to exhibit problems with long term memory, suggesting a function for PrPc . Stanley Prusiner, who discovered prions and coined the term, received the Nobel Prize in Medicine in 1997 for his work. I think that if I chanced to be on A protein making up a prion I'd twist it and for goodness sakes Stop it from making fold mistakes

Amyloids

Amyloids are a collection of improperly folded protein aggregates that are found in the human body. As a consequence of their misfolding, they are insoluble and contribute to some twenty human diseases including important neurological ones involving prions. Diseases include (affected protein in parentheses) - Alzheimer's disease (Amyloid β), Parkinson's disease (α-synuclein), Huntington's disease (huntingtin), rheumatoid arthritis (serum amyloid A), fatal familial insomnia (PrPSc), and others.

Amino acid sequence plays a role in amyloidogenesis. Glutamine-rich polypeptides are common in yeast and human prions. Trinucleotide repeats are important in Huntington's disease. Where sequence is not a factor, hydrophobic association between β-sheets can play a role.

Amyloid β

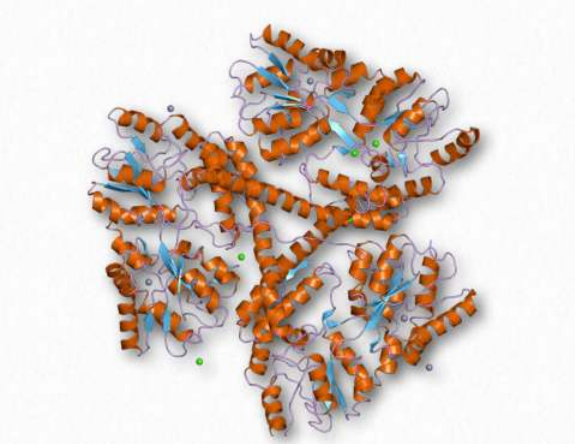


Figure 2.49 - Huntingtin

Amyloid β refers to collections of small proteins (36-43 amino acids) that appear to play a role in Alzheimer's disease. (Tau protein is the other factor.) They are, in fact, the main components of amyloid plaques found in the brains of patients suffering from the disease and arise from proteolytic cleavage of a larger amyloid precursor glycoprotein called Amyloid Precursor Protein, an integral membrane protein of nerve cells whose function is not known. Two proteases, β-secretase and γ- secretase perform this function. Amyloid β proteins are improperly folded and appear to induce other proteins to misfold and thus precipitate and form the amyloid characteristic of the disease. The plaques are toxic to nerve cells and give rise to the dementia characteristic of the disease.

It is thought that aggregation of amyloid β proteins during misfolding leads to generation of reactive oxygen species and that this is the means by which neurons are damaged. It is not known what the actual function of amyloid β is. Autosomal dominant mutations in the protein lead to early onset of the disease, but this occurs in no more than 10% of the cases. Strategies for treating the disease include inhibition of the secretases that generate the peptide fragments from the amyloid precursor protein.

Huntingtin

Huntingtin is the central gene in Huntington's disease. The protein made from it is glutamine rich, with 6-35 such residues in its wild-type form. In Huntington's disease, this gene is mutated, increasing the number of glutamines in the mutant protein to between 36 and 250. The size of the protein varies with the number of glutamines in the mutant protein, but the wild-type protein has over 3100 amino acids and a molecular weight of about 350,000 Da. Its precise function is not known, but huntingtin is found in nerve cells, with the highest level in the brain. It is thought to possibly play roles in transport, signaling, and protection against apoptosis. Huntingtin is also required for early embryonic development. Within the cell, huntingtin is found localized primarily with microtubules and vesicles.

Trinucleotide repeat

The huntingtin gene contains many copies of the sequence CAG (called trinucleotide repeats), which code for the many glutamines in the protein. Huntington's disease arises when extra copies of the CAG sequence are generated when the DNA of the gene is being copied. Expansion of repeated sequences can occur due to slipping of the polymerase relative to the DNA template during replication. As a result, multiple additional copies of the trinucleotide repeat may be made, resulting in proteins with variable numbers of glutamine residues. Up to 35 repeats can be tolerated without problem. The number of repeats can expand over the course of a person's lifetime, however, by the same mechanism. Individuals with 36-40 repeats begin to show signs of the disease and if there are over 40, the disease will be present.

Molecular chaperones

The importance of the proper folding of proteins is highlighted by the diseases associated with misfolded proteins, so it is no surprise, then, that cells expend energy to facilitate the proper folding of proteins. Cells use two classes of proteins known as molecular chaperones, to facilitate such folding in cells. Molecular chaperones are of two kinds, the chaperones, and the chaperonins. An example of the first category is the Hsp70 class of proteins. Hsp stands for "heat shock protein", based on the fact that these proteins were first observed in large amounts in cells that had been briefly subjected to high temperatures. Hsps function

to assist cells in stresses arising from heat shock and exposure to oxidizing conditions or toxic heavy metals, such as cadmium and mercury. However, they also play an important role in normal conditions, where they assist in the proper folding of polypeptides by preventing aberrant interactions that could lead to misfolding or aggregation. The Hsp70 proteins are found in almost all cells and use ATP hydrolysis to stimulate structural changes in the shape of the chaperone to accommodate binding of substrate proteins. The binding domain of Hsp70s contains a β-barrel structure which wraps around the polypeptide chain of the substrate and has affinity for hydrophobic side chains of amino acids. As shown in Figure 2.50, Hsp70 binds to polypeptides as they emerge from ribosomes during protein synthesis. Binding of substrate stimulates ATP hydrolysis and this is facilitated by another heat shock protein known as Hsp40. The hydrolysis of ATP causes the Hsp70 to taken on a closed conformation that helps shield exposed hydrophobic residues and prevent aggregation or local misfolding.

After protein synthesis is complete, ADP is released and replaced by ATP and this results in release of the substrate protein, which then allows the full length polypeptide to fold correctly.

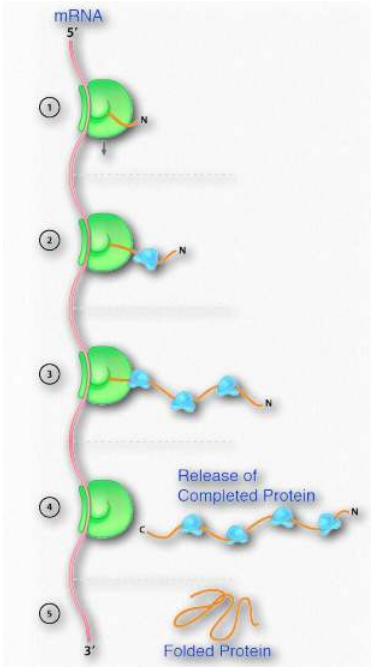


Figure 2.50 - Action of Hsp70 (blue) to facilitate proper folding of a protein (orange) Image by Aleia Kim

In heat shock

In times of heat shock or oxidative stress, Hsp70 proteins bind to unfolded hydrophobic regions of proteins to similarly prevent them from aggregating and allowing them to properly refold. When proteins are damaged, Hsp70 recruits enzymes that ubiquitinate the damaged protein to target them for destruction in proteasomes. Thus, the Hsp70 proteins play an important role in ensuring not only that proteins are properly folded, but that damaged or nonfunctional proteins are removed by degradation in the proteasome.

Chaperonins

A second class of proteins involved in assisting other proteins to fold properly are known as chaperonins. The best studied chaperonins are the GroEL/GroES complex proteins found in bacteria (Figure 2.51).

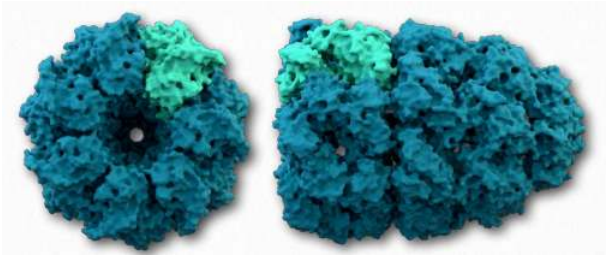


Figure 2.51 - View from bottom of GroEL (left) and GroEL/ GroES complex (right) Wikipedia

GroEL/GroES may not be able to undo aggregated proteins, but by facilitating proper folding, it provides competition for misfolding as a process and can reduce or eliminate problems arising from improper folding. GroEL is a double-ring 14mer with a hydrophobic region that can facilitate folding of substrates 15-60 kDa in size. GroES is a singlering heptamer that binds to GroEL in the presence of ATP and functions as a cover over GroEL. Hydrolysis of ATP by chaperonins induce large conformational changes that affect binding of substrate proteins and their folding. It is not known exactly how chaperonins fold proteins. Passive models postulate the chaperonin complex functioning inertly by preventing unfavorable intermolecular interactions or placing restrictions on spaces available for folding to occur. Active models propose that structural changes in the chaperonin complex induce structural changes in the substrate protein.

Protein breakdown

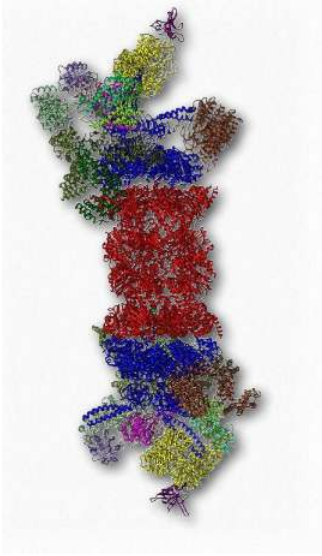


Figure 2.52 - 26S proteasome. Active site shown in red Wikipedia

Another protein complex that has an important function in the lifetime dynamics of proteins is the proteasome (Figure 2.52). Proteasomes, which are found in all eukaryotes and archaeans, as well as some bacteria, function to break down unneeded or damaged proteins by proteolytic degradation. Proteasomes help to regulate the concentration of some proteins and degrade ones that are misfolded. The proteasomal degradation pathway plays an important role in cellular processes that include progression through the cell cycle, modulation of gene expression, and response to oxidative stresses.

Degradation in the proteasome yields short peptides seven to eight amino acids in length. Threonine proteases play important roles. Breakdown of these peptides yields individual amino acids, thus facilitating their recycling in cells. Proteins are targeted for degradation in eukaryotic proteasomes by attachment to multiple copies of a small protein called ubiquitin (8.5 kDa - 76 amino acids). The enzyme catalyzing the reaction is known as ubiquitin ligase. The resulting polyubiquitin chain is bound by the proteasome and degradation begins. Ubiquitin was named due to it ubiquitously being found in eukaryotic cells.

Ubiquitin

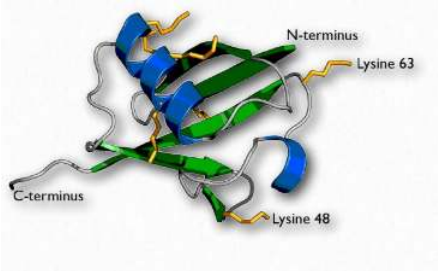


Figure 2.53 - Ubiquitin (lysine side chains shown in yellow) Wikipedia

Ubiquitin (Figure 2.53) is a small (8.5 kDa) multi-functional protein found in eukaryotic cells. It is commonly added to target proteins by action of ubiquitin ligase enzymes (E3 in Figure 2.54). One (ubiquitination) or many (polyubiquitination) ubiquitin

molecules may be added. Attachment of the ubiquitin is through the side chain of one of seven different lysine residues in ubiquitin.

The addition of ubiquitin to proteins has many effects, the best known of which is targeting the protein for degradation in the proteasome. Proteasomal targeting is seen when polyubiquitination occurs at lysines #29 and 48. Polyubiquitination or monoubiquitination at other lysines can result in altered cellular location and changed protein-protein interactions. The latter may alter affect inflammation, endocytic trafficking, translation and DNA repair.

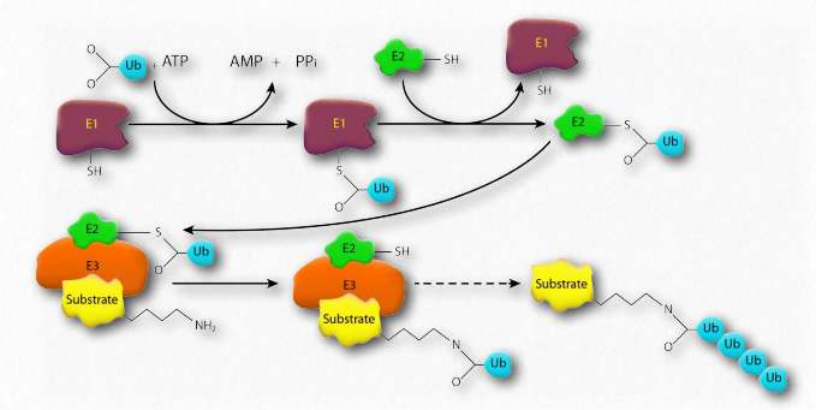
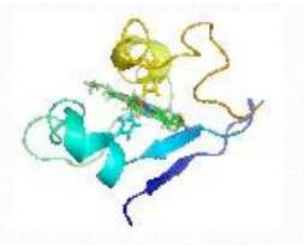


Figure 2.54 - Pathway for ubiquitination of a target substrate protein Image by Pehr Jacobson

Ubiquitin ligase malfunction

Parkin is a Parkinson's disease-related protein that, when mutated, is linked to an inherited form of the disease called autosomal recessive juvenile Parkinson's disease. The function of the protein is not known, but it is a component of the E3 ubiquitin ligase system responsible for transferring ubiquitin from the E2 protein to a lysine side chain on the target protein. It is thought that mutations in parkin lead to proteasomal dysfunction and a consequent inability to break down proteins harmful to dopaminergic neurons. This results in the death or malfunction of these neurons, resulting in Parkinson's disease.

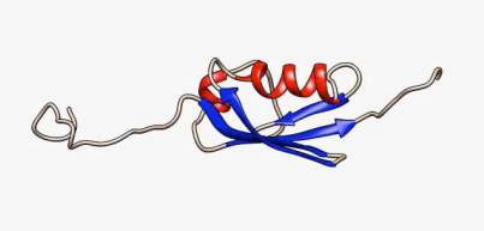
Intrinsically disordered proteins



Movie 2.1 - Dynamic movement of cytochrome C in solution Wikipedia

As is evident from the many examples described elsewhere in the book, the 3-D structure of proteins is important for their function. But, increasingly, it is becoming evident that not all proteins fold into a stable structure. Studies on the so-called intrinsically disordered proteins (IDPs) in the past cou- ple of decades has shown that many proteins are biologically active, even thought they fail to fold into stable structures. Yet other proteins exhibit regions that remain unfolded (IDP regions) even as the rest of the polypeptide folds into a structured form.

Intrinsically disordered proteins and disordered regions within proteins have, in fact, been known for many years, but were regarded as an anomaly. It is only recently, with the realization that IDPs and IDP regions are widespread among eukaryotic proteins, that it has been recognized that the observed disorder is a "feature, not a bug".



Movie 2.2 SUMO-1, a protein with intrinsically disordered sections Wikipedia

Comparison of IDPs shows that they share sequence characteristics that appear to favor their disordered state. That is, just as some amino acid sequences may favor the folding of a polypeptide into a particular structure, the amino acid sequences of IDPs favor their remaining unfolded. IDP regions are seen to be low in hydrophobic residues and unusually rich in polar residues and proline. The presence of a large number of charged amino acids in the IDPs can inhibit folding through charge repulsion, while the lack of hydrophobic residues makes it difficult to form a stable hydrophobic core, and proline discourages the formation of helical structures. The observed differences between amino acid sequences in IDPs and structured proteins have been used to design algorithms to predict whether a given amino acid sequence will be disordered.

What is the significance of intrinsically disordered proteins or regions? The fact that this property is encoded in their amino acid sequences suggests that their disorder may be linked to their function. The flexible, mobile nature of some IDP regions may play a crucial role in their function, permitting a transition to a folded structure upon binding a protein partner or undergoing post-translational modification. Studies on several wellknown proteins with IDP regions suggest some answers. IDP regions may enhance the ability of proteins like the lac repressor to translocate along the DNA to search for specific binding sites. The flexibility of IDPs can also be an asset in protein-protein interactions, especially for proteins that are known to interact with many different protein partners.

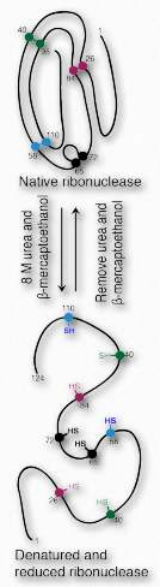


Figure 2.55 - Denaturation and renaturation of ribonuclease Wikipedia

For example, p53 has IDP regions that may allow the protein to interact with a variety of functional partners. Comparison of the known functions of proteins with predictions of disorder in these proteins suggests that IDPs and IDP regions may disproportionately function in signaling and regulation, while more structured proteins skew towards roles in catalysis and transport. Interestingly, many of the proteins found in both ribosomes and spliceosomes are predicted to have IDP regions that may play a part in correct assembly of these complexes. Even though IDPs have not been studied intensively for very long, what little is known of them suggests that they play an important and underestimated role in cells.

Metamorphic proteins

Another group of proteins that have recently changed our thinking about protein structure and function are the so-called metamorphic proteins. These proteins are capable of forming more than one stable, folded state starting with a single amino acid sequence. Although it is true that multiple folded conformations are not ruled out by the laws of physics and chemistry, metamorphic proteins are a relatively new discovery. It was known, of course, that prion proteins were capable of folding into alternative structures, but metamorphic proteins appear to be able to toggle back and forth between two stable structures. While in some cases, the metamorphic protein undergoes this switch in response to binding another molecule, some proteins that can accomplish this transition on their own. An interesting example is the signaling molecule, lymphotactin. Lymphotactin has two biological functions that are carried out by its two conformers- a monomeric form that binds the lymphotactin receptor and a dimeric form that binds heparin. It is possible that this sort of switching is more widespread than has been thought.

Refolding denatured proteins

All information for protein folding is contained in the amino acid sequence of the protein. It may seem curious then that most proteins do not fold into their proper, fully active form after they have been+++ denatured and the denaturant is removed. A few do, in fact. One good example is bovine ribonuclease (Figure 2.55). Its catalytic activity is very resistant to heat and urea and attempts to denature it don't work very well. However, if one treats the enzyme with β-mercaptoethanol (which breaks disulfide bonds) prior to urea treatment and/or heating, activity is lost, indicating that the covalent disulfide bonds help stabilize the overall enzyme structure and when they are broken, denaturation can readily occur. When the mixture cools back down to room temperature, over time some enzyme activity reappears, indicating that ribonuclease re-folded under the new conditions.

Interestingly, renaturation will occur maximally if a tiny amount of β-mercaptoethanol is left in the solution during the process. The reason for this is because β- mercaptoethanol permits reduction (and breaking) of accidental, incorrect disulfide bonds during the folding process. Without it, these disulfide bonds will prevent proper folds from forming.

Irreversible denaturation

Most enzymes, however, do not behave like bovine ribonuclease. Once denatured, their activity cannot be recovered to any significant There are not very many ways Inactivating RNase It's stable when it's hot or cold Because disulfides tightly hold If you desire to make it stall Use hot mercaptoethanol extent. This may seem to contradict the idea of folding information being inherent to the sequence of amino acids in the protein. It does not.

Most enzymes don't refold properly after denaturation for two reasons. First, normal folding may occur as proteins are being made. Interactions among amino acids early in the synthesis are not "confused" by interactions with amino acids later in the synthesis because those amino acids aren't present as the process starts.

Chaperonins' role

In other cases, the folding process of some proteins in the cell relied upon action of chaperonin proteins. In the absence of chaperonins, interactions that might result in misfolding occur, thus preventing proper folding. Thus, early folding and the assistance of chaperonins eliminate some potential "wrong-folding" interactions that can occur if the entire sequence was present when folding started.

Quaternary structure

A fourth level of protein structure is that of quaternary structure. It refers to structures that arise as a result of interactions between multiple polypeptides. The units can be identical multiple copies or can be different polypeptide chains. Adult hemoglobin is a good example of a protein with quaternary structure, being composed of two identical chains called α and two identical chains called β.

Though the α-chains are very similar to the β- chains, they are not identical. Both of the α- and the β-chains are also related to the single polypeptide chain in the related protein called myoglobin. Both myoglobin and hemoglobin have similarity in binding oxygen, but their behavior towards the molecule differ significantly. Notably, hemoglobin's multiple subunits (with quaternary structure) compared to myoglobin's single subunit (with no quaternary structure) give rise to these differences.

References

1. https://en.wikipedia.org/wiki/Van_der_W aals_force 105

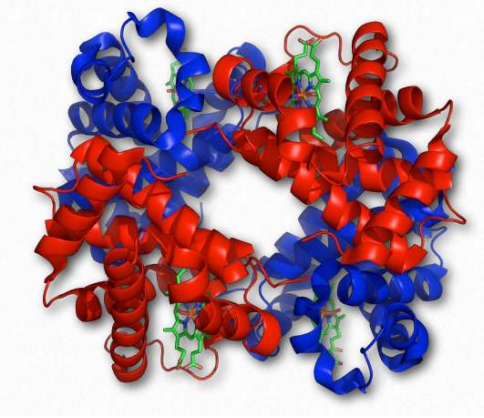
This page titled 2.2: Structure and Function- Proteins I is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **2.3: Structure & Function- Proteins I** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

2.3: Structure and Function- Proteins II

Source: BiochemFFA_2_3.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

In this section, we hope to bring to life the connection between structure and function of proteins. So far, we have described notable features of the four elements (primary, secondary, tertiary, and quaternary) of protein structure and discussed example proteins/motifs exhibiting them. In this section, we will examine from a functional perspective a few proteins/domains whose function relies on secondary, tertiary, or quaternary structure. It is, of course a bit of a narrow focus to ascribe protein function to any one component of structure, but our hope is by presenting these examples, we can bring to life the way in which a protein's secondary, tertiary, and quarternary structure lead to the functions it has.



Hemoglobin Wikipedia

Fibrous proteins - secondary structure

Proteins whose cellular or extracellular roles have a strong structural component are composed primarily of primary and second structure, with little folding of the chains. Thus, they have very little tertiary structure and are fibrous in nature. Proteins exhibiting these traits are commonly insoluble in water and are referred to as fibrous proteins (also called scleroproteins). The examples described in this category are found exclusively in animals where they serve roles in flesh, connective tissues and hardened external structures, such as hair. They also contain the three common fibrous protein structures α -helices (keratins), β-strands/sheets (fibroin & elastin) and triple helices (collagen). The fibrous proteins have some commonality of amino acid sequence. Each possesses an abundance of repeating sequences of amino acids with small, non-reactive side groups. Many contain short repeats of sequences, often with glycine.

Keratins



Figure 2.56 - The horns of an impala are composed of keratin Wikipedia

The keratins are a family of related animal proteins that take numerous forms. α-keratins are structural components of the outer layer of human skin and are integral to hair, nails, claws, feathers, beaks, scales, and hooves. Keratins provide strength to tissues, such as the tongue, and over 50 different keratins are encoded in the human genome. At a cellular level, keratins comprise the intermediate filaments of the cytoskeleton. α- keratins primarily contain α-helices, but can also have β-strand/sheet structures. Individual α-helices are often intertwined to form coils of coiled structures and these strands can also be further joined together by disulfide bonds, increasing structural strength considerably. This is particularly relevant for α-keratin in hair, which contains about 14% cysteine. The odor of burned hair and that of the chemicals used to curl/uncurl hair (breaking/re-making disulfide bonds) arise from their sulfurous components. β-keratins are comprised of β-sheets, as their name implies.

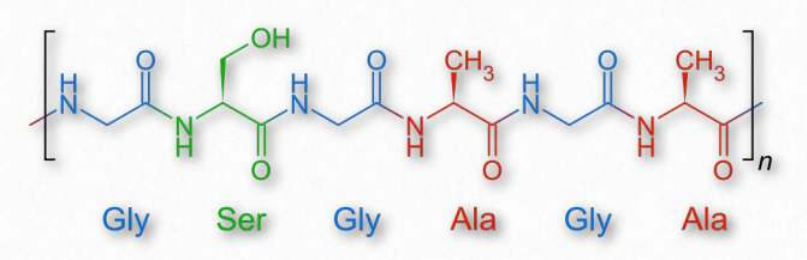


Figure 2.57 - The repeating amino acid sequence of fibroin

Fibroin

An insoluble fibrous protein that is a component of the silk of spiders and the larvae of moths and other insects, fibroin is comprised of antiparallel β-strands tightly packed together to form β- sheets. The primary structure of fibroin is a short repeating sequence with glycine at every other residue (Figure 2.57). The small R-groups of the glycine and alanine in the repeating sequence allows for the tight packing characteristic of the fibers of silk. Wikipedia link HERE Elastin As suggested by its name, elastin is a protein with elastic characteristics that functions in many tissues of the body to allow them to resume their shapes after expanding or contracting. The protein is rich in glycine and proline and can comprise over 50% of the weight of dry, defatted arteries.


Figure 2.58 - Weaving of a silk sari Wikipedia

Elastin

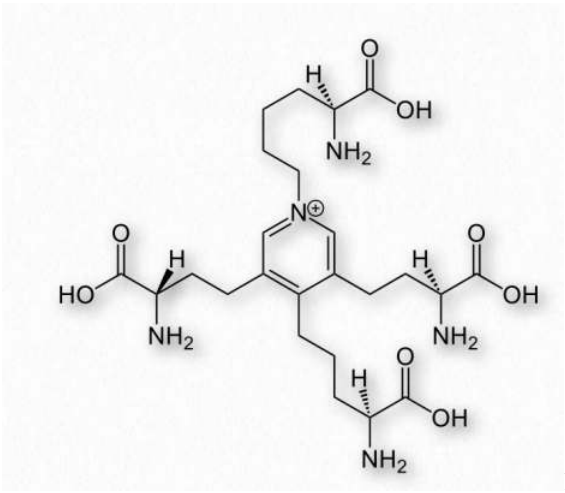
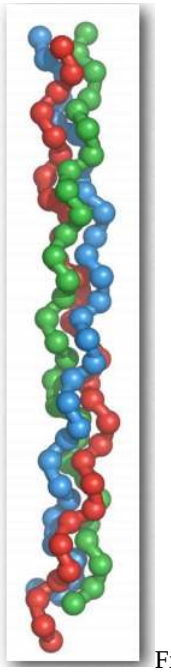


Figure 2.59 - Desmosine Wikipedia

is made by linking tropoelastin proteins together through lysine residues to make a durable complex crosslinked by desmosine. In arteries, elastin helps with pressure wave propagation for facilitating blood flow.

Collagen


Figure 2.60 - Collagen's triple helix Wikipedia

Collagen is the most abundant protein in mammals, occupying up to a third of the total mass. There are at least 16 types of collagen. Its fibers are a major component of tendons and they are also found abundantly in skin. Collagen is also prominent in cornea, cartilage, bone, blood vessels and the gut.

Collagen's structure is an example of a helix of helices, being composed of three lefthanded helical chains that each are coiled together in a right-handed fashion to make the collagen fiber (Figure 2.60). Each helix is stretched out more than an α-helix, giving it an extended appearance. On the inside of the triple helical structure, only residues of glycine are found, since the side chains of other amino acids are too bulky. Collagen chains have the repeating structure glycinem-n where m is often proline and n is often hydroxyproline (Figure 2.61).

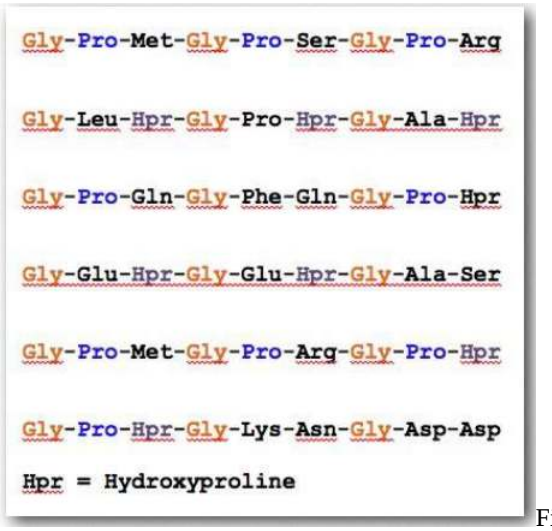


Figure 2.61 - Repeating sequences in collagen

Collagen is synthesized in a pre-procollagen form. Processing of the pre-procollagen in the endoplasmic reticulum results in glycosylation, removal of the 'pre' sequence, and hydroxylation of lysine and proline residues (see below). The hydroxides can form covalent cross-links with each other, strengthening the collagen fibers. As pro-collagen is exported out of the cell, proteases trim it, resulting in a final form of collagen called tropocollagen.

Hydroxylation

Hydroxylation of proline and lysine side chains occurs post-translationally in a reaction catalyzed by prolyl-4-hydroxylase and lysyl-hydroxylase (lysyl oxidase), respectively. The reaction requires vitamin C. Since hydroxylation of these residues is essential for formation of stable triple helices at body temperature, vitamin C deficiency results in weak, unstable collagen and, consequently, weakened connective tissues. It is the cause of the disease known as scurvy. Hydrolyzed collagen is used to make gelatin, which is important in the food industry. collagens. Wikipedia link HERE

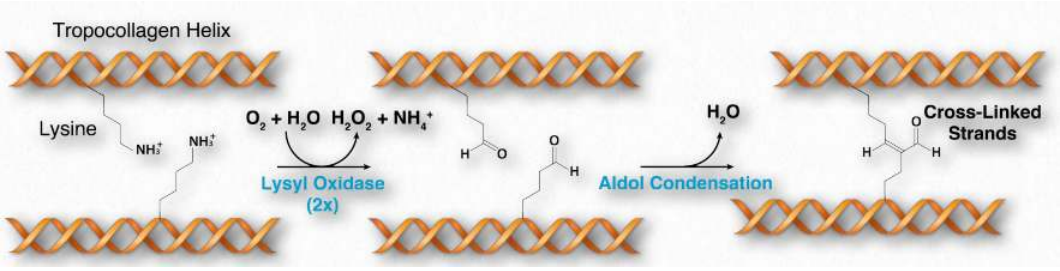


Figure 2.62 - Oxidation and cross-linking of lysine residues in tropocollagen. Only two strands of the triple helix are shown for simplicity Image by Aleia Kim

Lamins

Lamins are fibrous proteins that provide structure in the cell nucleus and play a role in transcription regulation. They are similar to proteins making up the intermediate filaments, but have extra amino acids in one coil of the protein. Lamins help to form the nuclear lamin in the interior of the nuclear envelope and play important roles in assembling and disassembling the latter in the process of mitosis. They also help to position nuclear pores. In the process of mitosis, disassembly of the nuclear envelope is promoted by phosphorylation of lamins by a protein called mitosis promoting factor and assembly is favored by reversing the reaction (dephosphorylation).

Structural domains - tertiary structure

Every globular protein relies on its tertiary structure to perform its function, so rather than trying to find representative proteins for tertiary structure (an almost impossible task!), we focus here on a few elements of tertiary structure that are common to many proteins. These are the structural domains and they differ from the structural motifs of supersecondary structure by being larger (25-500 amino acids), having a conserved amino acid sequence, and a history of evolving and functioning independently of the

protein chains they are found in. Structural domains are fundamental units of tertiary structure and are found in more than one protein. A structural domain is selfstabilizing and often folds independently of the rest of the protein chain.

Leucine zipper

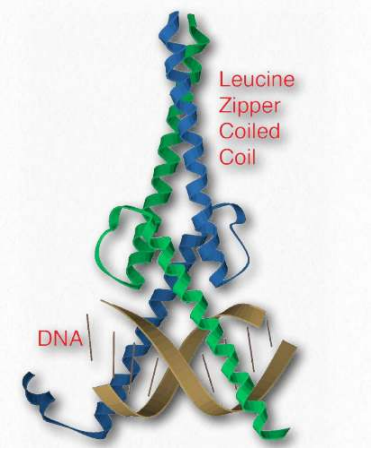
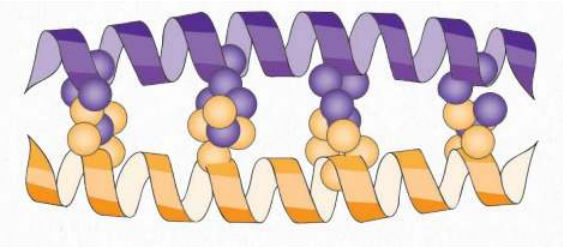


Figure 2.63 - Leucine zipper bound to DNA Wikipedia

A common feature of many eukaryotic DNA binding proteins, leucine zippers are characterized by a repeating set of leucine residues in a protein that interact like a zipper to favor dimerization. Another part of the domain has amino acids (commonly arginine and lysine) that allow it to interact with the DNA double helix (Figure 2.63). Transcription factors that contain leucine zippers include Jun-B, CREB, and AP-1 fos/ jun.


Figure 2.64 - Leucine zipper structure. Leucines are indicated by orange and purple balls. Image by Penelope Irving

Zinc fingers

The shortest structural domains are the zinc fingers, which get their name from the fact that one or more coordinated zinc ions stabilize their finger-like structure. Despite their name, some zinc fingers do not bind zinc. There are many structural domains classified as zinc fingers and these are grouped into different families. Zinc fingers were first identified as components of DNA binding transcription factors, but others are now known to bind RNA, protein, and even lipid structures. Cysteine and histidine side chains commonly play roles in coordinating the zinc.

Src SH_2 domain

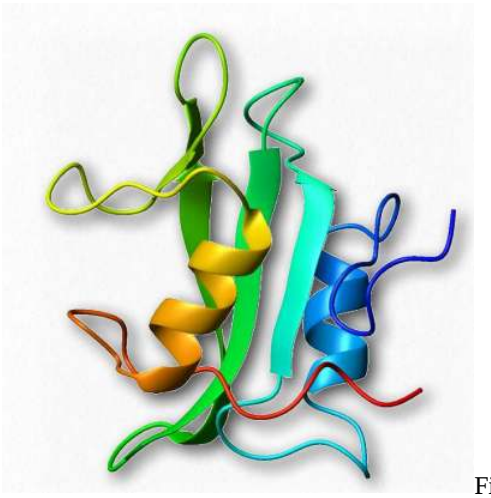


Figure 2.65 - SH2 Domain Wikipedia

The Src oncoprotein contains a conserved SH_2structural domain that recognizes and binds phosphorylated tyrosine side chains in other proteins (Figure 2.65). Phosphorylation is a fundamental activity in signaling and phosphorylation of tyrosine and interaction between proteins carrying signals is critically needed for cellular communication. The SH_2domain is found in over 100 human proteins.

Helix-turn-helix domain

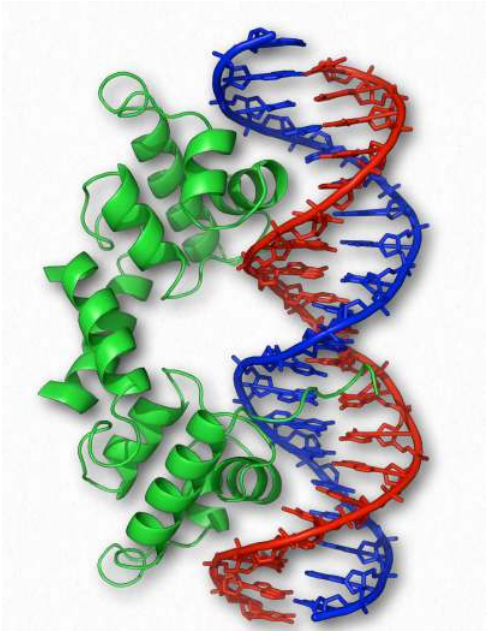


Figure 2.66 - Helix-Turn-Helix Domain of a Protein Bound to DNA Wikipedia

Helix-turn-helix is a common domain found in DNA binding proteins, consisting of two α-helices separated by a small number of amino acids. As seen in Figure 2.66, the helix parts of the structural domain interact with the bases in the major groove of DNA. Individual α-helices in a protein are part of a helix-turn-helix structure, where the turn separates the individual helices.

Pleckstrin homology domain

Pleckstrin Homology (PH) domains are protein domains with important functions in the process of signaling. This arises partly from the affinity for binding phosphorylated inositides, such as PIP2 and PIP3, found in Figure 2.66 - Helix-Turn-Helix Domain of a Protein Bound to DNA Wikipedia Figure 2.65 - SH2Domain Wikipedia biological membranes. PH domains can also bind to G-proteins and protein kinase C. The domain spans about amino acids and is found in numerous signaling proteins. These include Akt/Rac Serine/ Threonine Protein Kinases, Btk/ltk/Tec tyrosine protein kinases, insulin receptor substrate (IRS-1), Phosphatidylinositolspecific phospholipase C, and several yeast proteins involved in cell cycle regulation.

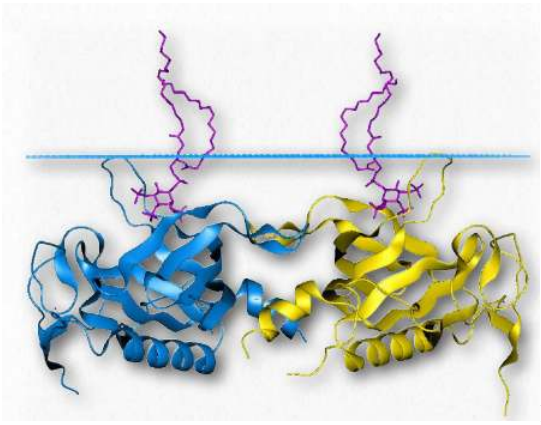


Figure 2.67 - Pleckstrin homology domain of Btk tyrosine protein kinase. The protein is embedded in a membrane (above blue line) Wikipedia

Structural globular proteins

Enzymes catalyze reactions and proteins such as hemoglobin perform important specialized functions. Evolutionary selection has reduced and eliminated waste so that we can be sure every protein in a cell has a function, even though in some cases we may not know what it is. Sometimes the structure of the proFigure 2.68 - Relationship of basement membrane to epithelium, endothelium, and connective tissue tein is its primary function because the structure provides stability, organization, connections other important properties. It is with this in mind that we present the following proteins.

Basement membrane

The basement membrane is a layered extracellular matrix of tissue comprised of protein fibers (type IV collagen) and glycosaminoglycans that separates the epithelium from other tissues (Figure 2.68). More importantly, the basement membrane acts like a glue to hold tissues together. The skin, for example, is anchored to the rest of the body by the basement membrane. Basement membranes provide an interface of interaction between cells and the environment around them, thus facilitating signaling processes. They play roles in differentiation during embryogenesis and also in maintenance of function in adult organisms.

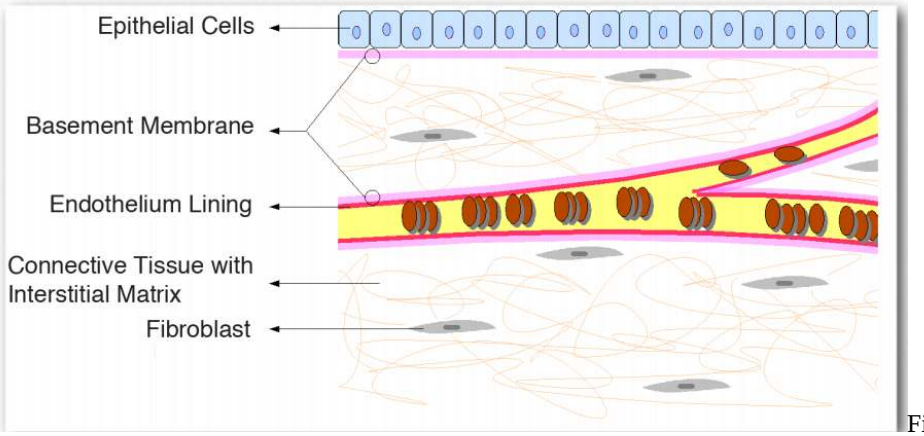


Figure 2.68 - Relationship of basement membrane to epithelium, endothelium, and connective tissue

Actin

Actin is the most abundant globular protein found in most types of eukaryotic cells, comprising as much as 20% of the weight of muscle cells. Similar proteins have been identified in bacteria (MreB) and archaeons (Ta0583). Actin is a monomeric subunit able to polymerize readily into two different types of filaments. Microfilaments are major component of the cytoskeleton and are acted on by myosin in the contraction of muscle cells (See HERE). Actin will be discussed in more detail in the next section HERE.

Intermediate Filaments

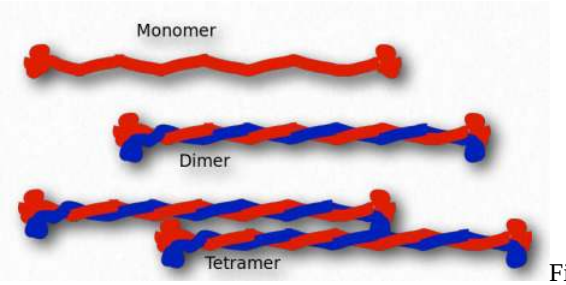


Figure 2.69 - Assembly of intermediate filaments

Intermediate filaments are a part of the cytoskeleton in many animal cells and are comprised of over 70 different proteins. They are called intermediate because their size (average diameter = 10 nm) is between that of the microfilaments (7 nm) and the microtubules (25 nm).

The intermediate filament components include fibrous proteins, such as the keratins and the lamins, which are nuclear, as well as cytoplasmic forms. Intermediate filaments give flexibility to cells because of their own physical properties. They can, for example, be stretched to several times of their original length.

Six types

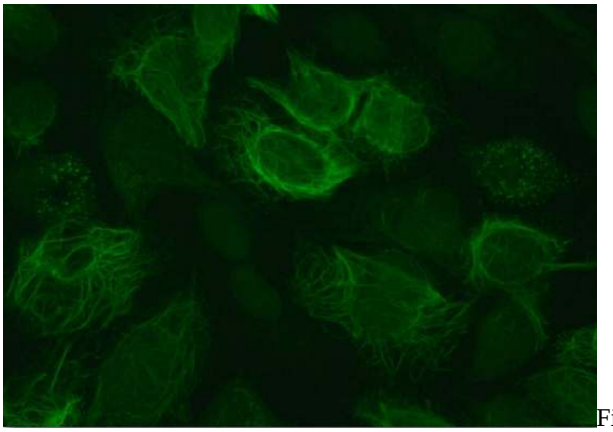
There are six different types of intermediate filaments. Type I and II are acidic or basic and attract each other to make larger filaments. They include epithelial keratins and trichocytic keratins (hair components). Type III proteins include four structural proteins - desmin, GFAP (glial fibrillary acidic protein), peripherin, and vimentin. Type IV also is a grouping of three proteins and one multiprotein structure (neurofilaments). The three proteins are α-internexin, synemin, and syncoilin. Type V intermediate filaments encompass the lamins, which give structure to the nucleus. Phosphorylation of lamins leads to their disassembly and this is important in the process of mitosis. The Type VI category includes only a single protein known as nestin.

Tubulin

A third type of filament found in cells is that of the microbutules. Comprised of a polymer of two units of a globular protein called tubulin, microtubules provide “rails” for motor proteins to move organelles and other “cargo” from one part of a cell to another. Microtubules and tubulin are discussed in more detail HERE.

Vimentin

Vimentin (Figure 2.70) is the most widely distributed protein of the intermediate filaments. It is expressed in fibroblasts, leukocytes, and blood vessel endothelial cells. The protein has a significant role maintaining the position of organelles in the cytoplasm, with attachments to the nucleus, mitochondria, and endoplasmic reticulum (Figure 2.70). Vimentin provides elasticity to cells and resilience that does not arise from the microtubules or microfilaments. Wounded mice that lack the vimentin gene survive, but take longer to heal wounds than wild type mice. Vimentin also controls the movement of cholesterol from lysosomes to the site of esterification. The result is a reduction in the amount of cholesterol stored inside of cells and has implications for adrenal cells, which must have esters of cholesterol.


Figure 2.70 - Vimentin in cells Wikipedia

Mucin

Mucins are a group of proteins found in animal epithelial tissue that have many glycosyl residues on them and typically are of high molecular weight (1 to 10 million Da). They are gel-like in their character and are often used for lubrication. Mucus is comprised of mucins. In addition to lubrication, mucins also help to control mineralization, such as bone formation in vertebrate organisms and calcification in echinoderms. They also play roles in the immune system by helping to bind pathogens. Mucins are commonly secreted onto mucosal surfaces (nostrils, eyes, mouth, ears, stomach, genitals, anus) or into fluids, such as saliva. Because of their extensive mucosylation, mucins hold a considerable amount of water (giving them the “slimy” feel) and are resistant to proteolysis.

Vinculin

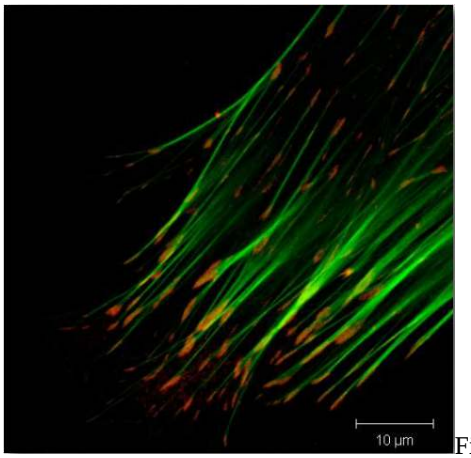
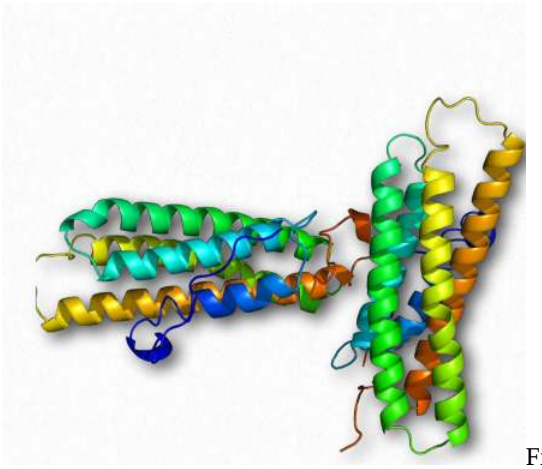


Figure 2.71 - Actin filaments (green) attached to vinculin in focal adhesion (red) Wikipedia

Vinculin (Figure 2.72) is a membrane cytoskeletal protein found in the focal adhesion structures of mammalian cells. It is found at cell-cell and cell-matrix junctions and interacts with integrins, talin, paxillins and F-actin. Vinculin is thought to assist (along with other proteins) in anchoring actin microfilaments to the membrane (Figure 2.71). Binding of vinculin to actin and to talin is regulating by polyphosphoinositides and can be inhibited by acidic phospholipids.


Figure 2.72 - Vinculin Wikipedia

Syndecans

Syndecans are transmembrane proteins that make a single pass with a long amino acid chain (24-25 residues) through plasma membranes and facilitate G proteincoupled receptors’ interaction with Figure 2.71 - Actin filaments (green) attached to vinculin in focal adhesion (red) Wikipedia ligands, such as growth factors, fibronectin, collagens (I, III, and IV) and antithrombin-1. Syndecans typically have 3-5 heparan sulfate and chondroitin sulfate chains attached to them.

Heparan sulfate can be cleaved at the site of a wound and stimulate action of fibroblast growth factor in the healing process. The role of syndecans in cell-cell adhesion is shown in mutant cells lacking syndecan I that do not adhere well to each other. Syndecan 4 is also known to adhere to integrin. Syndecans can also inhibit the spread of tumors by the ability of the syndecan 1 ectodomain to suppress growth of tumor cells without affecting normal epithelial cells.

Defensin

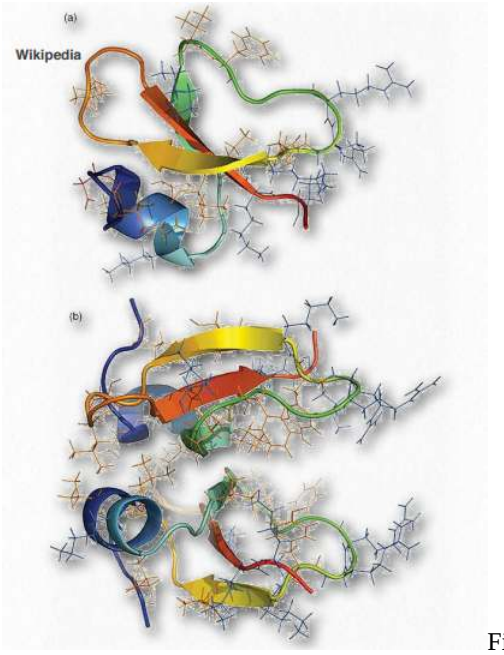


Figure 2.73 - Defensin monomer (top) and dimer (bottom) - Cationic residues in blue, hydrophobic residues in orange, and anionic residues in red Wikipedia

Defensins (Figure 2.73) are a group of small cationic proteins (rich in cysteine residues) that serve as host defense peptides in vertebrate and invertebrate organisms. They protect against infection by various bacteria, fungi, and viruses. Defensins contain between 18 and 45 amino acids with (typically) about 6- 8 cysteine residues. In the immune system, defensins help to kill bacteria engulfed by phagocytosis by epithelial cells and neutrophils. They kill 120 Figure 2.72 - Vinculin Wikipedia bacteria by acting like ionophores - binding the membrane and opening pore-like structures to release ions and nutrients from the cells.

Focal adhesions

In the cell, focal adhesions are structures containing multiple proteins that mechanically link cytoskeletal structures (actin bundles) with the extracellular matrix. They are dynamic, with proteins bringing and leaving with signals regarding the cell cycle, cell motility, and more almost constantly. Focal adhesions serve as anchors and as a signaling hub at cellular locations where integrins bind molecules and where membrane clustering events occur. Over 100 different proteins are found in focal adhesions.

Focal adhesions communicate important messages to cells, acting as sensors to update information about the status of the extracellular matrix, which, in turn, adjusts/ affects their actions. In sedentary cells, they are stabler than in cells in motion because when cells move, focal adhesion contacts are established at the “front” and removed at the rear as motion progresses. This can be very important in white blood cells’ ability to find tissue damage.

Ankyrin

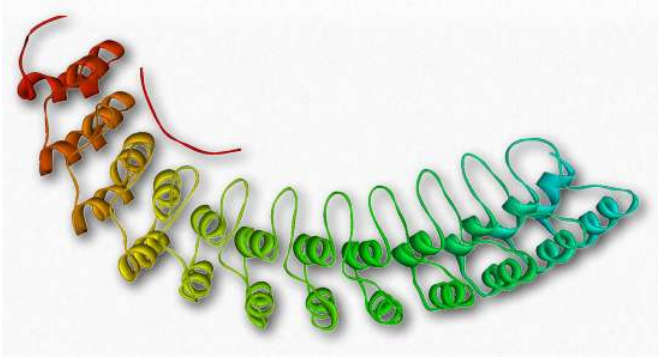


Figure 2.74 - Ankyrin’s membrane-binding domain

Ankyrins (Figure 2.74) are a family of membrane adaptor proteins serving as “anchors” to interconnect integral membrane proteins to the spectrin-actin membrane cytoskeleton. Ankyrins are anchored to the plasma membrane by covalently linked palmitoyl-CoA. They bind to the β subunit of spectrin and at least a dozen groups of integral membrane proteins. The ankyrin proteins contain four functional domains: an N-terminal region with 24 tandem ankyrin repeats, a central spectrin-binding domain, a “death domain” interacting with apoptotic proteins, and a C-terminal regulatory domain that is highly varied significantly among different ankyrins.

Spectrin

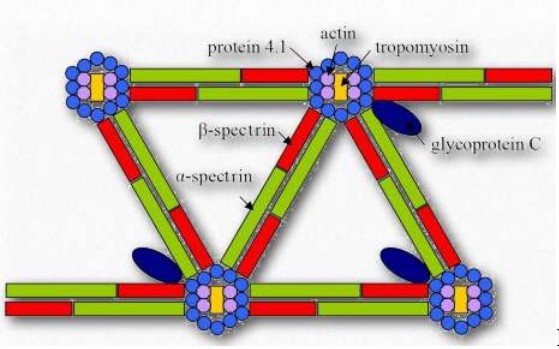


Figure 2.75 - Spectrin and other proteins in the cytoskeleton Wikipedia

Spectrin (Figures 2.75 & 2.76) is a protein of the cellular cytoskeleton that plays an important role in maintaining its structure and the integrity of the plasma membrane. In animals, spectrin gives red blood cells their shape. Spectrin is located inside the inner layer of the eukaryotic plasma membrane where it forms a network of pentagonal or hexagonal arrangements.

Spectrin fibers collect together at junctional complexes of actin and is also attached to ankyrin for stability, as well as numerous integral membrane proteins, such as glycophorin.

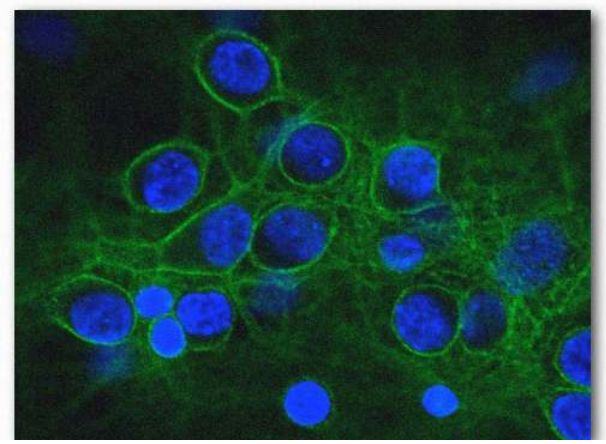


Figure 2.76 - Spectrin (green) and nuclei (Blue) Wikipedia

Integrins

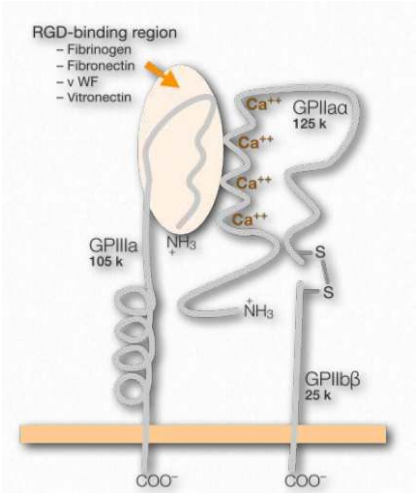


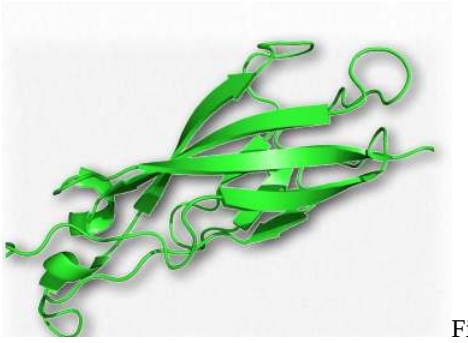
Figure 2.77 - Integrin and its binding site (on top left) Wikipedia

In multicellular organisms, cells need connections, both to each other and to the extracellular matrix. Facilitating these attachments at the cellular end are transmembrane proteins known as integrins (Figure 2.77). Integrins are found in all metazoan cells. Ligands for the integrins include collagen, fibronectin, laminin, and vitronectin. Integrins function not only in attachment, but also in communication, cell migration, virus linkages (adenovirus, for example), and blood clotting. Integrins are able to sense chemical and mechanical signals about the extracellular matrix and move that information to intracellular domains as part of the process of signal transduction. Inside the cells, responses to the signals affect cell shape, regulation of the cell cycle, movement, or changes in other cell receptors in the membrane. The process is dynamic and allows for rapid responses as may be necessary, for example in the process of blood clotting, where the integrin known as GPIbIIIa (on the surface of blood platelets) attaches to fibrin in a clot as it develops.

Integrins work along with other receptors, including immunoglobulins, other cell adhesion molecules, cadherins, selectins, and syndecans. In mammals the proteins have a large number of subunits - 18 α- and 8 β-chains. They are a bridge between its links outside the cell to the extracellular matrix (ECM) and its links inside the cell to the cytoskeleton. Integrins play central role in formation and stability of focal adhesions. These are large molecular complexes arising from clustering of integrin-ECM connections. In the process of cellular movement, integrins at the “front” of the cell (in the direction of the movement), make new attachments to substrate and release connections to substrate in the back of the cell. These latter integrins are then endocytosed and reused.

Integrins also help to modulate signal transduction through tyrosine kinase receptors in the cell membrane by regulating movement of adapters to the plasma membrane. β1c integrin, for example, recruits the Shp2 phosphatase to the insulin growth factor receptor to cause it to become dephosphorylated, thus turning off the signal it communicates. Integrins can also help to recruit signaling molecules inside of the cell to activated tyrosine kinases to help them to communicate their signals.

Cadherins


Figure 2.78 - Extracellular ectodomain of a cadherin

Cadherins (Figure 2.78) constitute a type-1 class of transmembrane proteins playing important roles in cell adhesion. They require calcium ions to function, forming adherens junctions that hold tissues together (See Figure 2.69). Cells of a specific cadherin type will preferentially cluster with each other in preference to associating with cells containing a different cadherin type. Caderins are

both receptors and places for ligands to attach. They assist in the proper positioning of cells in development, separation of different tissue layers, and cell migration.

Selectins



Figure 2.79 - Selectin bound to a sugar Wikipedia

Selectins (Figure 2.79) are cell adhesion glycoproteins that bind to sugar molecules. As such, they are a type of lectin - proteins that bind sugar polymers (see HERE also). All selectins have an N-terminal calcium-dependent lectin domain, a single transmembrane domain, and an intracellular cytoplasmic tail.

There are three different types of selectins, 1) E-selectin (endothelial); 2) L (lymphocytic; and 3) P (platelets and endothelial cells. Selectins function in lymphocyte homing (adhesion of blood lymphocytes to cells in lymphoid organs), in inflammation processes, and in cancer metastasis. Near the site of inflammation, P-selectin on the surface of blood capillary cells interacts with glycoproteins on leukocyte cell surfaces. This has the effect of slowing the movement of the leukocyte. At the target site of inflammation, E- selectin on the endothelial cells of the blood vessel and L-selectin on the surface of the leukocyte bind to their respective carbohydrates, stopping the leukocyte movement. The leukocyte then crosses the wall of the capillary and begins the immune response. Selectins are involved in the inflammatory processes of asthma, psoriasis, multiple scleroris, and rheumatoid arthritis.

Laminins

Laminins are extracellular matrix glycoproteins that a major components of the basal lamina and affect cell differentiation, migration, and adhesion. They are secreted into the extracellular matrix where they are incorporated and are essential for tissue maintenance and survival. When laminins are defective, muscles may not form properly and give rise to muscular dystrophy.

Laminins are associated with fibronectin, entactin, and perlecan proteins in type IV collagen networks and bind to integrin receptors in the plasma membrane. As a consequence, laminins contribute to cellular attachment, differentiation, shape, and movement. The proteins are trimeric in structure, having one α-chain, a β-chain, and a γ-chain. Fifteen combinations of different chains are known.

Vitronectin

Vitronectin is a glycoprotein (75kDa) found in blood serum (platelets), the extracellular matrix, and in bone. It promotes the process of cell adhesion and spreading and binds to several protease inhibitors (serpins). It is secreted from cells and is believed to play roles in blood clotting and the malignancy of tumors. One domain of vitronectin binds to plasminogen activator inhibitor and acts to stabilize it. Another domain of the protein binds to cellular integrin proteins, such as the vitronectin receptor that anchors cells to the extracellular matrix.

Catenins

Catenins are a family of proteins interacting with cadherin proteins in cell adhesion (Figure 2.69). Four main types of catenins are known, α-, β-, γ-, and δ-catenin. Catenins play roles in cellular organization before development occurs and help to regulate cellular growth. α-catenin and β-catenin are found at adherens junctions with cadherin and help cells to maintain epithelial layers. Cadherins are connected to actin filaments of the cytoskeleton and catenins play the critical role. Catenins are important for the process whereby cellular division is inhibited when cells come in contact with each other (contact inhibition).

When catenin genes are mutated, cadherin cell adhesions can disappear and tumorigenesis may result. Catenins have been found to be associated with colorectal and numerous other forms of cancer.

Glycophorins

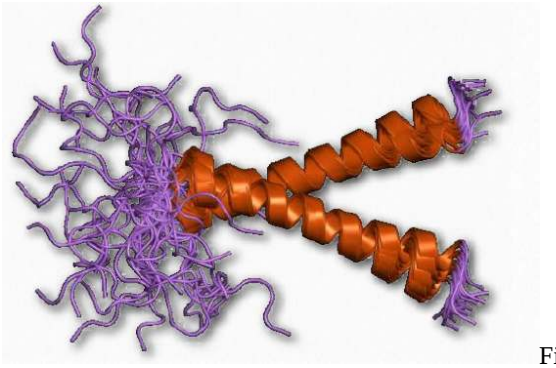


Figure 2.80 - Glycophorin a

All of the membrane proteins described so far are notable for the connections they make to other proteins and cellular structures. Some membrane proteins, though, are designed to reduce cellular connections to proteins of other cells. This is particularly important for blood cells where “stickiness” is undesirable except where clotting is concerned.

Glycophorins (Figure 2.80) are membrane-spanning sialoglycoproteins of red blood cells. They are heavily glycosylated (60%).and rich in sialic acid, giving the cells a very hydrophilic (and negatively charged) coat, which enables them to circulate in the bloodstream without adhering to other cells or the vessel walls.

Five glycophorins have been identified - four (A,B,C,and D) from isolated membranes and a fifth form (E) from coding in the human genome. The proteins are abundant, forming about 2% of the total membrane proteins in these cells. Glycophorins have important roles in regulating RBC membrane mechanical properties and shape. Because some glycophorins can be expressed in various nonerythroid tissues (particularly Glycophorin C), the importance of their interactions with the membrane skeleton may have a considerable biological significance.

Cooperativity and allosterism - quaternary structure

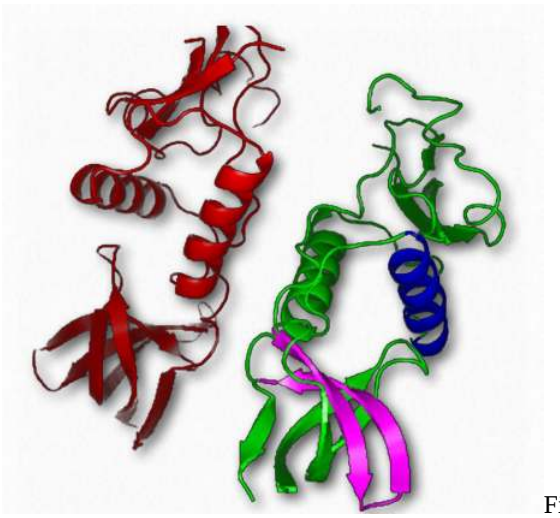


Figure 2.81 - Two polypeptide units of a protein interact in quaternary structure Wikipedia

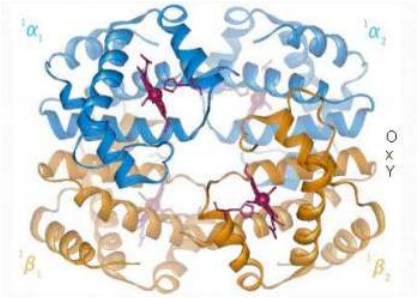
Quaternary structure, of course describes the interactions of individual subunits of a multi-subunit protein (Figure 2.81). The result of these interactions can give rise to important biological phenomena, such as cooperative binding of substrates to a protein and allosteric effects on the action of an enzyme.

Allosteric effects can occur by a series of mechanisms, but a common feature is that binding of an effector to an enzyme subunit causes (or locks) the enzyme in either a Tstate (less activity) or an R-state (more activity). Effectors can be enzyme substrates (homotropic effectors) or non-substrates (heterotropic effectors). Allosterism will be covered in more depth in the Catalysis chapter HERE.

We begin our consideration of quaternary structure with a discussion of cooperativity, how it arises in the multi-subunit protein hemoglobin and how its properties contrast with those of the related, single subunit protein myoglobin.

Cooperativity

Cooperativity is defined as the phenomenon where binding of one ligand molecule by a protein favors the binding of additional molecules of the same type. Hemoglobin, for example, exhibits cooperativity when the binding of an oxygen molecule by the iron of the heme group in one of the four subunits causes a slight conformation change in the subunit. This happens because the heme iron is attached to a histidine side chain and binding of oxygen 'lifts' the iron along with the histidine ring (also known as the imidazole ring).



Movie 2.3 - Hemoglobin's structural changes on binding oxygen Wikipedia

Since each hemoglobin subunit interacts with and influences the other subunits, they too are induced to change shape slightly when the first subunit binds to oxygen (a transition described as going from the T-state to the R-state). These shape changes favor each of the remaining subunits binding oxygen, as well. This is very important in the lungs where oxygen is picked up by hemoglobin, because the binding of the first oxygen molecule facilitates the rapid uptake of more oxygen molecules. In the tissues, where the oxygen concentration is lower, the oxygen leaves hemoglobin and the proteins flips from the R-state back to the Tstate.

CO_2 transport

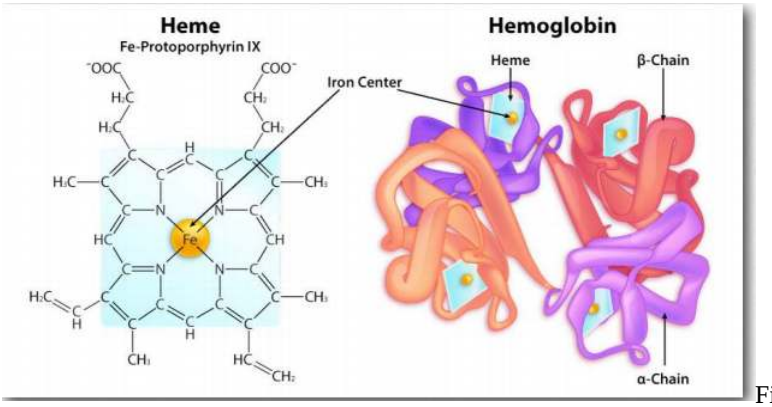


Figure 2.82 - Heme structure within hemoglobin Image by Aleia Kim

Cooperativity is only one of many fascinating structural aspects of hemoglobin that help the body to receive oxygen where it is needed and pick it up where it is abundant. Hemoglobin also assists in the transport of the product of cellular respiration (carbon dioxide) from the tissues producing it to the lungs where it is exhaled. Like the binding of oxygen to hemoglobin, binding of other

molecules to hemoglobin affects its affinity for oxygen. The effect is particularly pronounced when comparing the oxygen binding characteristics of hemoglobin's four subunits with the oxygen binding of the related protein myoglobin's single subunit (Figure 2.83).

Different oxygen binding

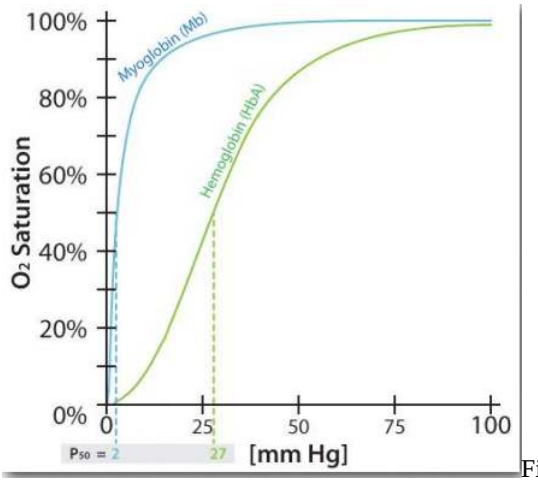


Figure by Aleia Kim

Like hemoglobin, myoglobin contains an iron in a heme group that binds to oxygen. The structure of the globin protein in myoglobin is very similar to the structure of the globins in hemoglobin and hemoglobin is thought to have evolved from myoglobin in evolutionary history. As seen in Figure 2.83, the binding curve of hemoglobin for oxygen is S-shaped (sigmoidal), whereas the binding curve for myoglobin is hyperbolic. What this tells us is that hemoglobin's affinity for oxygen is low at a low concentration oxygen, but increases as the oxygen concentration increases. Since myoglobin very quickly saturates with oxygen, even under low oxygen concentrations, it says that its affinity for oxygen is high and doesn't change.

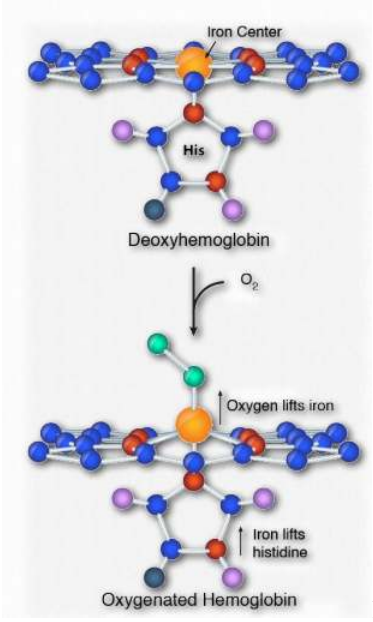
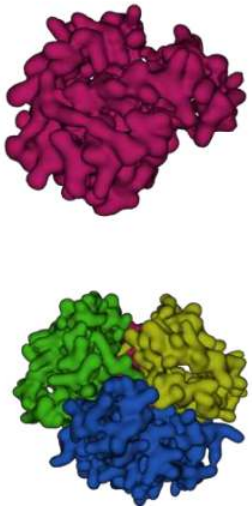


Figure 2.84 - Binding of oxygen at the heme center of hemoglobin Image by Aleia Kim

Because myoglobin has only a single subunit, binding of oxygen by that subunit can't affect any other subunits, since there are no other subunits to affect. Consequently, cooperativity requires more than one subunit. Therefore, hemoglobin can exhibit cooperativity, but myoglobin can't. It is worth noting that simply having multiple subunits does not mean cooperativity will exist. Hemoglobin is one protein that exhibits the characteristic, but many multisubunit proteins do not.



Interactive 2.2 - Hemoglobin in the presence (top) and absence (bottom) of oxygen

Storage vs. delivery

The lack of ability of myoglobin to adjust its affinity for oxygen according to the oxygen concentration (low affinity at low oxygen concentration, such as in tissues and high affinity at high oxygen concentration, such as in the lungs) means it is better suited for storing oxygen than for delivering it according to the varying oxygen needs of and animal body. As we shall see, besides cooperativity, hemoglobin has other structural features that allow it to deliver oxygen precisely where it is needed most in the body.

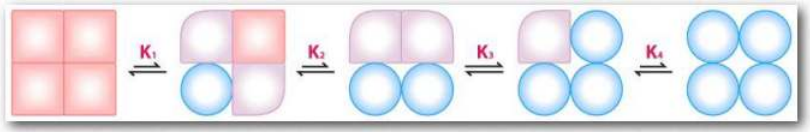


Figure 2.85 - Sequential model of binding. The sequential model is one way to explain hemoglobin's cooperativity. Squares represent no oxygen bound. Circles represent subunits bound with oxygen and rounded subunits correspond to units whose affinity for oxygen increases by interacting with a subunit that has bound oxygen. Image by Aleia Kim 131

Bohr effect

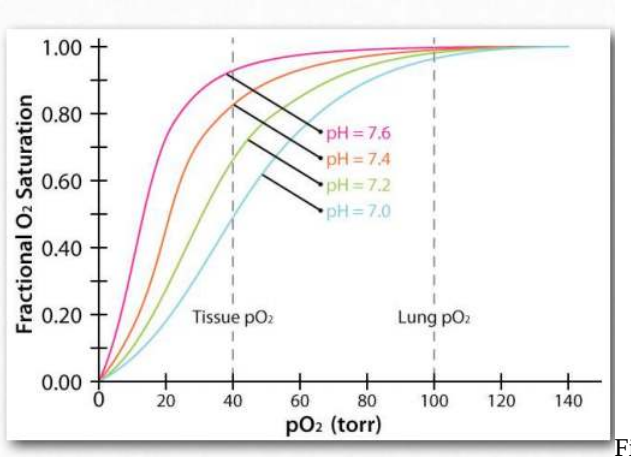


Figure 2.86 - The Bohr effect with respect to pH changes Image by Aleia Kim

The Bohr Effect was first described over 100 years ago by Christian Bohr, father of the famous physicist, Niels Bohr. Shown graphically (Figures 2.86, 2.87, and 2.88), the observed effect is that hemoglobin's affinity for oxygen decreases as the pH decreases and as the concentration of carbon dioxide increases. Binding of the protons and carbon dioxide by amino Figure 2.85 - Sequential model of binding. The sequential model is one way to explain hemoglobin's cooperativity. Squares represent no oxygen bound. Circles represent subunits bound with oxygen and rounded subunits correspond to units whose affinity for oxygen increases

by interacting with a subunit that has bound oxygen. Image by Aleia Kim acid side chains in the globin proteins helps to facilitate structural changes in them. Most commonly, the amino acid affected by protons is histidine #146 of the β strands. When this happens, the ionized histidine can form an ionic bond with the side chain of aspartic acid #94, which has the effect of stabilizing the T-state (reduced oxygen binding state) and releasing oxygen. Other histidines and the amine of the amino terminal amino acids in the α-chains are also binding sites for protons.

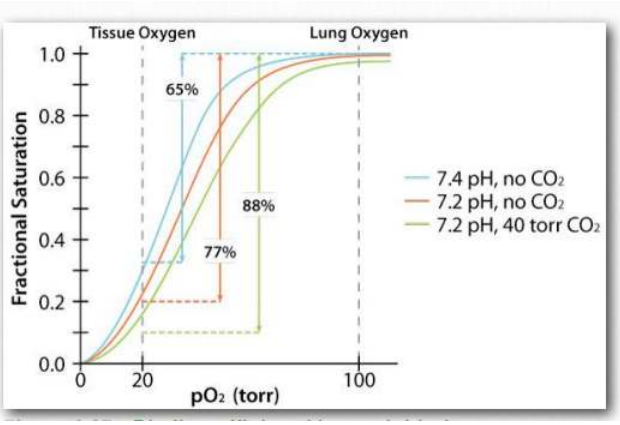


Figure 2.87 - Binding affinity of hemoglobin for oxygen under different conditions Image by Aleia Kim

2,3-BPG

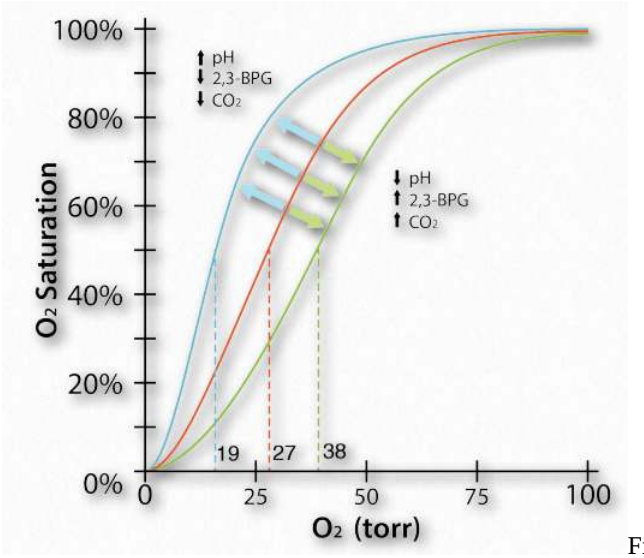


Figure 2.88 - The Bohr effect physiologically - oxygen binding curves for resting muscle (blue), active muscle (green) and reference muscle (orange) with respect to pH, 2,3-BPG, and CO2 Image by Aleia Kim

Another molecule favoring the release of oxygen by hemoglobin is 2,3- bisphosphoglycerate (also called 2,3-BPG or just BPG - Figure 2.89). Like protons and carbon dioxide, 2,3-BPG is produced by actively respiring tissues, as a byproduct of glucose metabolism. The 2,3-BPG mole cule fits into the 'hole of the donut' of adult hemoglobin (Figure 2.89). Such binding of 2,3-BPG favors the T-state (tight - low oxygen binding) of hemoglobin, which has a reduced affinity for oxygen. In the absence of 2,3-BPG, hemoglobin can more easily exist in the R-state (relaxed - higher oxygen binding), which has a high affinity for oxygen.

Smokers

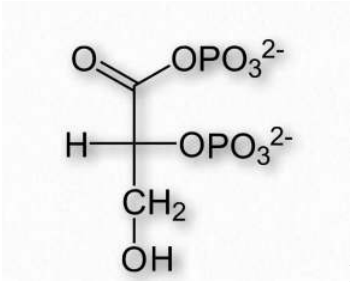


Figure 2.89 - The structure of 2,3 bisphosphoglycerate (2,3-BPG)

Notably, the blood of smokers is higher in the concentration of 2,3-BPG than non-smokers, so more of their hemoglobin remains in the T-state and thus the oxygen carrying capacity of smokers is lower than non-smokers.Another reason why smokers' oxygen carrying capacity is lower than that of non-smokers is that cigarette smoke contains carbon monoxide and this molecule, which has almost identical dimensions to molecular oxygen, effectively outcompetes with oxygen for binding to the iron atom of heme (Figure 2.90). Part of carbon monoxide's toxicity is due to its ability to bind hemoglobin and prevent oxygen from binding.

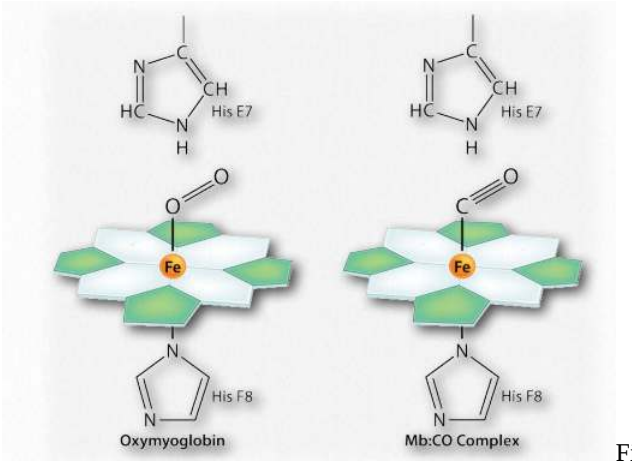


Figure 2.90 - Binding of oxygen (left) and carbon monoxide (right) by a heme group of hemoglobin Image by Aleia Kim

Carbon dioxide

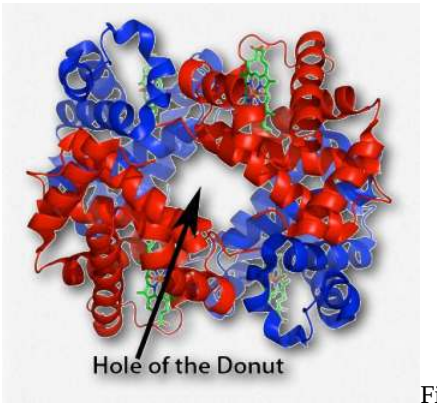


Figure 2.91 - Hemoglobin's hole of the donut for binding 2,3-BPG Wikipedia

Carbon dioxide binds to form a carbamate when binding the α-amine of each globin chain. The process of forming this structure releases a proton, which helps to further enhance the Bohr effect. Physiologically, the binding of CO2 and H+ has significance because actively respiring tissues (such as contracting muscles) require oxygen and release protons and carbon dioxide. The higher the concentration of protons and carbon dioxide, the more oxygen is released to feed the tissues that need it most.

About 40% of the released protons and about 20% of the carbon dioxide are carried back to the lungs by hemoglobin. The remainder travel as part of the bicarbonate buffering system or as dissolved CO2. In the lungs, the process reverses itself. The lungs have a higher pH than respiring tissues, so protons are released from hemoglobin and CO2 too is freed to be exhaled.

Fetal hemoglobin

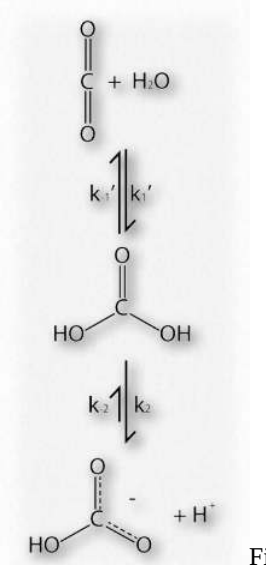


Figure 2.92 - Formation of bicarbonate from CO2 in blood

Adult hemoglobin releases oxygen when it binds 2,3- BPG. This is in contrast to fetal hemoglobin, which has a slightly different configuration (α2γ2) than adult hemoglobin (α2β2). Fetal hemoglobin has a greater affinity for oxygen than maternal hemoglobin, allowing the fetus to obtain oxygen effectively from the mother's blood. Part of the reason for fetal hemoglobin's greater affinity for oxygen is that it doesn't bind 2,3-BPG. Consequently, fetal hemoglobin remains in the R-state much more than adult hemoglobin and because of this, fetal hemoglobin has greater affinity for oxygen than adult hemoglobin and can take oxygen away from adult hemoglobin. Thus, the fetus can get oxygen from the mother.

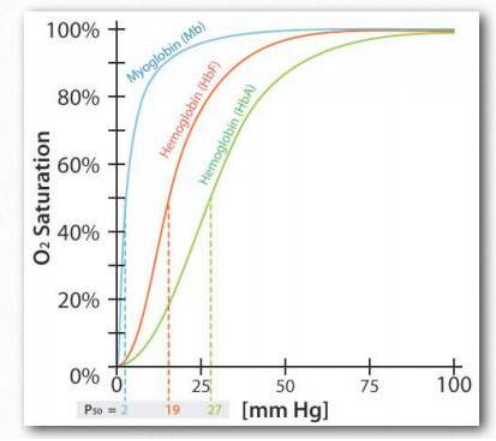


Figure 2.93 - Comparison of oxygen binding of myoglobin (blue), fetal hemoglobin (orange), and adult hemoglobin (green) Image by Aleia Kim

Sickle cell disease

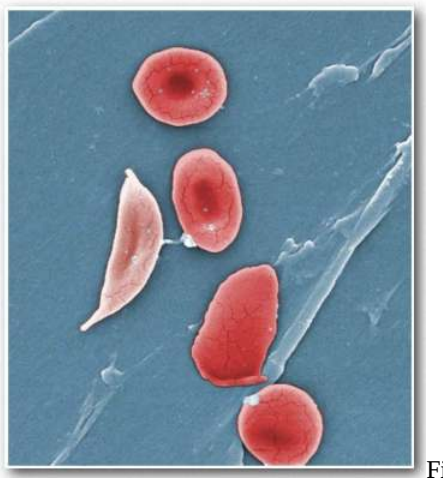


Figure 2.94 - Four normal red blood cells (right) and one sickled red blood cell (left) Wikipedia

Mutations to the globin genes coding for hemoglobin can sometimes have deleterious consequences. Sickle cell disease (also called sickle cell anemia) is a genetically transmitted disease that arises from such mutations. There are different forms of the disease. It is a recessive trait, meaning that to be afflicted with it, an individual must inherit two copies of the mutated gene.

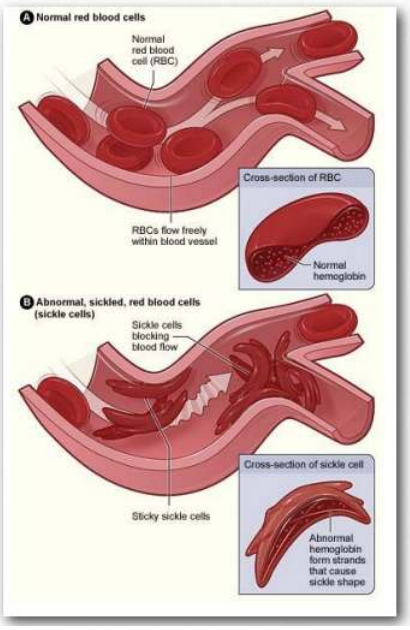


Figure 2.95 - Movement of blood in capillaries. Top - normal red blood cells. Bottom - sickled red blood cells

The predominant form of hemoglobin in adults is hemoglobin A, designated HbA (two α chains and two β chains). The mutant form is known as HbS. The most common mutation is an A to T mutation in the middle of the codon for the seventh amino acid (some counting schemes call it the sixth amino acid) of the β-chain. This results in conversion of a GAG codon to GTG and thus changes the amino acid specified at that position from a glutamic acid to a valine. This minor change places a small hydrophobic patch of amino acids on the surface of the β-globin chains.

Polymerization

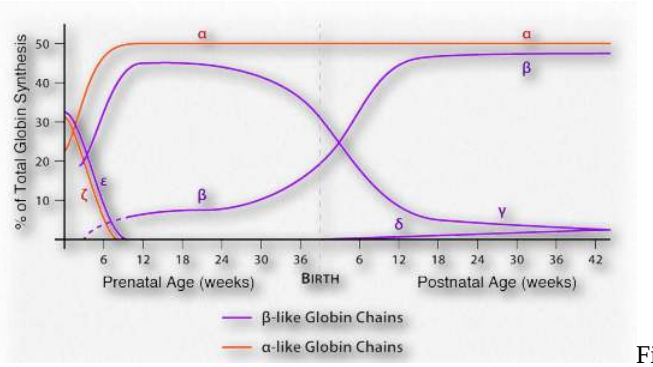


Figure 2.96 - Pattern of expression of six different globins of hemoglobin - α,β,γ,ε,δ, and ζ Image by Aleia Kim

Under conditions of low oxygen, these hydrophobic patches will associate with each other to make long polymers of hemoglobin molecules. The result is that the red blood cells containing them will change shape from being rounded to forming the shape of a sickle (Figure 2.94). Rounded red blood cells readily make it through tiny capillaries, but sickleshaped cells do not.

Worse, they block the flow of other blood cells. Tissues where these blockages occur are already low in oxygen, so stopping the flow of blood through them causes them to go quickly anaerobic, causing pain and, in some cases, death of tissue. In severe circumstances, sickled red blood cells death may result. The disease is referred to as an anemia because the sickling of the red blood cells targets them for removal by the blood monitoring system of the body, so a person with the disease has chronically reduced numbers of red blood cells.

Heterozygote advantage

Interestingly, there appears to be a selective advantage to people who are heterozygous for the disease in areas where malaria is prominent. Heterozygotes do not suffer obvious ill effects of the disease, but their red blood cells appear to be more susceptible to rupture when infected. As a consequence, the parasite gets less of a chance to reproduce and the infected person has a greater chance of survival.

The protective effect of the mutant gene, though, does not extend to people who suffer the full blown disease (homozygotes for the mutant gene). Treatments for the disease include transfusion, pain management, and avoidance of heavy exertion. The drug hydroxyurea has been linked to reduction in number and severity of attacks, as well as an increase in survival time1,2. It appears to work by reactivating expression of the fetal hemoglobin gene, which typically is not synthesized to any significant extent normally after about 6 weeks of age.

Oxygen binding

Animals have needs for oxygen that differ from all other organisms. Oxygen, of course, is the terminal electron acceptor in animals and is necessary for electron transport to work. When electron transport is functioning, ATP generation by cells is many times more efficient than when it is absent. Since abundant ATP is essential for muscular contraction and animals move around a lot - to catch prey, to exercise, to escape danger, etc., having an abundant supply of oxygen is important.

This is particularly a concern deep inside tissues where diffusion of oxygen alone (as occurs in insects) does not deliver sufficient quantities necessary for long term survival. The issue is not a problem for plants since, for the most part, their motions are largely related to growth and thus don’t have rapidly changing needs/demands for oxygen that animals have. Unicellular organisms have a variety of mechanisms for obtaining oxygen and surviving without it. Two other important oxygen binding proteins besides hemoglobin are myoglobin and hemocyanin.

Myoglobin

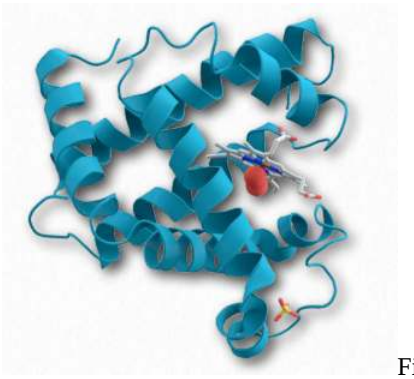


Figure 2.97 - Myoglobin bound to oxygen

Myoglobin is the primary oxygen-storage protein found in animal muscle tissues. In contrast to hemoglobin, which circulates throughout the body, myoglobin protein is only found in muscle tissue and appears in the blood only after injury. Like hemoglobin, myoglobin binds oxygen at a prosthetic heme group it contains.

The red color of meat arises from the heme of myoglobin and the browning of meat by cooking it comes from oxidation of the ferrous (Fe++) ion of myoglobin's heme to the ferric (Fe+++) ion via oxidation in the cooking process. As meat sits in our atmosphere (an oxygen-rich environment), oxidation of Fe++ to Fe+++ occurs, leaving the brown color noted above. If meat is stored in a carbon monoxide (CO) environment, CO binds to the heme group and reduces the amount of oxidation, keeping meat looking red for a longer period of time.

High affinity

Myoglobin (Figure 2.97) displays higher affinity for oxygen at low oxygen concentrations than hemoglobin and is therefore able to absorb oxygen delivered by hemoglobin under these conditions. Myoglobin's high affinity for oxygen makes it better suited for oxygen storage than delivery. The protein exists as a single subunit of globin (in contrast to hemoglobin, which contains four subunits) and is related to the subunits found in hemoglobin. Mammals that dive deeply in the ocean, such as whales and seals, have muscles with particularly high abundance of myoglobin. When oxygen concentration in muscles falls to low levels, myoglobin releases its oxygen, thus functioning as an oxygen "battery" that delivers oxygen fuel when needed and holding onto it under all other conditions. Myoglobin holds the distinction of being the first protein for which the 3D structure was determined by X-ray crystallography by John Kendrew in 1958, an achievement for which he later won the Nobel Prize.

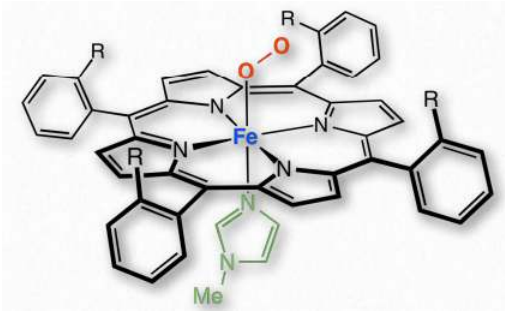


Figure 2.98 - Oxygen bound at heme of myoglobin Wikipedia

Hemocyanin

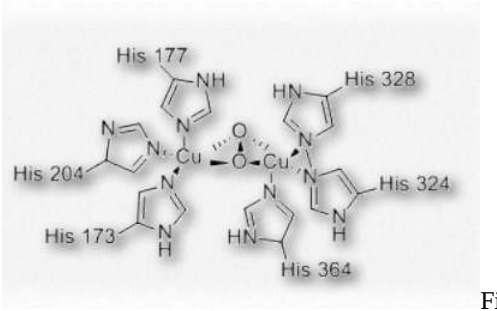


Figure 2.99 - Oxygen binding in hemocyanin Wikipedia

Hemocyanin is the protein transporting oxygen in the bodies of molluscs and arthropods. It is a coppercontaining protein found not within blood cells of these organisms, but rather is suspended in the circulating hemolymph they possess. The oxygen binding site of hemocyanin contains a pair of copper(I) cations directly coordinated to the protein by the imidazole rings of six histidine side chains.



Figure 2.100 - Hemocyanin (purple) in a red rock crab Wikipedia

Most, but not all hemocyanins bind oxygen non-cooperatively and are less efficient than hemoglobin at transporting oxygen. Notably, the hemocyanins of horseshoe crabs and some other arthropods do, in fact, bind oxygen cooperatively. Hemocyanin contains many subunit proteins, each with two copper atoms that can bind one oxygen molecule (O2). Subunit proteins have atomic masses of about 75 kilodaltons (kDa). These may be arranged in dimers or hexamers depending on species. Superstructures comprised of dimer or hexamer complexes are arranged in chains or clusters and have molecular weights of over 1500 kDa.

This page titled 2.3: Structure and Function- Proteins II is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **2.4: Structure and Function- Proteins II** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

CHAPTER OVERVIEW

3: Catalysis

3.1: Basic Principles of Catalysis

3.2: Control of Enzymatic Activity

3.3: Mechanisms of Catalysis

3: Catalysis is shared under a not declared license and was authored, remixed, and/or curated by LibreTexts.

3.1: Basic Principles of Catalysis

A printable version of this section is here: BiochemFFA_4_1.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

If there is a magical component to life, an argument can surely be made for it being catalysis. Thanks to catalysis, reactions that can take hundreds of years to complete in the uncatalyzed “real world,” occur in seconds in the presence of a catalyst. Chemical catalysts, such as platinum, can speed reactions, but enzymes (which are simply super-catalysts with a “twist,” as we shall see) put chemical catalysts to shame (Figure 4.1). To understand enzymatic catalysis, it is necessary first to understand energy. Chemical reactions follow the universal trend of moving towards lower energy, but they often have a barrier in place that must be overcome. The secret to catalytic action is reducing the magnitude of that barrier.

Enzyme	Nonenzymatic Half-Life	Uncatalyzed Rate (k_{un} s^{-1})	Catalyzed Rate (k_{cat} s^{-1})	Rate Enhancement (k_{cat} s^{-1}/k_{un} s^{-1})
OMP decarboxylase	78,000,000 years	28×10^{-16}	39	1.4×10^{17}
Staphylococcal nuclease	130,000 years	1.7×10^{-13}	95	5.6×10^{14}
Carboxypeptidase A	7.3 years	3.0×10^{-9}	578	1.9×10^{11}
Ketosteroid isomerase	7 weeks	1.7×10^{-7}	66,000	3.9×10^{11}
Triose phosphate isomerase	1.9 days	4.3×10^{-6}	4,300	1.0×10^{9}
Chorismate mutase	7.4 hours	2.6×10^{-5}	50	1.9×10^{6}
Carbonic anhydrase	5 seconds	1.3×10^{-1}	1×10^{6}	7.7×10^{6}

Abbreviations:
OMP - Orotine monophosphate
AMP - Adenosine monophosphate

Figure 4.1 - Rate enhancement for several enzymes Image by Aleia Kim (note: k is a rate constant with units 1/second)

Before discussing enzymes, it is appropriate to pause and discuss an important concept relating to chemical/biochemical reactions. That concept is *equilibrium* and it is very often misunderstood. The “equi" part of the word relates to equal, as one might expect, but it does not relate to absolute concentrations. What happens when a biochemical reaction is at equilibrium is that the concentrations of reactants and products do not change over time. This does not mean that the reactions have stopped. Remember that reactions are reversible, so there is a forward reaction and a reverse reaction: if you had 8 molecules of A, and 4 of B at the beginning, and 2 molecules of A were converted to B, while 2 molecules of B were simultaneously converted back to A, the number of molecules of A and B remain unchanged, i.e., the reaction is at equilibrium. However, you will notice that this does not mean that there are equal numbers of A and B molecules.

Concentration Matters

Contrary to the perceptions of many students, the concentrations of products and reactants at equilibrium are not equal to each other. The one exception to the previous statement occurs the ΔG°’ for a reaction is zero. Recall that ΔG° is Gibbs free energy change at standard state (1M concentrations of reactants and products), while ΔG°’ refers to the Gibbs free energy change for modified standard state (at pH 7, rather than 0, which would occur is proton concentration was 1M).

When ΔG°’ is zero,

$$\Delta G = RT \ln \left(\frac{[\text{Products}]}{[\text{Reactants}]} \right) \tag{3.1.1}$$

Because ΔG itself is zero at equilibrium, then

$$[Products] = [Reactants]. \tag{3.1.2}$$

This is the only circumstance where

$$[Products] = [Reactants] \tag{3.1.3}$$

at equilibrium. Reiterating, at equilibrium, the concentrations of reactant and product do not change over time. That is, for a reaction $A \rightleftharpoons B[A]$ at time zero when equilibrium is reached, $[A]_{T_0}$, will be the same 5 minutes later (assuming A and B are chemically stable). Thus,

$$[A]_{T_0} = [A]_{T+5} \tag{3.1.4}$$

Similarly,

$$[B]_{T_0} = [B]_{T+5} \tag{3.1.5}$$

For that matter, at any amount of time X after equilibrium has been reached,

$$[A]_{T_0} = [A]_{T+5} = [A]_{T_X} \tag{3.1.6}$$

and

$$[B]_{T_0} = [B]_{T+5} = [B]_{T_X} \tag{3.1.7}$$

However, unless ΔG°' = 0, it is wrong to say that at equilibrium, the concentration of products equals the concentration of reactants.

As we study biochemical reactions and reaction rates, it is important to remember that

1) reactions do not generally start at equilibrium;

2) all reactions move in the direction of equilibrium; and

3) reactions in cells behave just like those in test tubes - they do not begin at equilibrium, but they move towards it.

The reactions occurring in cells, though, are very dynamic and complex. In a test tube, they can be studied one at a time. In cells, the product of one reaction is often the substrate for another one. Reactions in cells are interconnected in this way, giving rise to what are called metabolic pathways. There are, in fact, thousands of different interconnected reactions going on continuously in cells.

Attempts to study a single reaction in a cell is daunting to say the least. For this reason, biochemists isolate enzymes from cells and study reactions individually. It is with this in mind that we begin our consideration of the phenomenon of catalysis by describing, first, the way in which enzymes work.

Activation energy

Figure 4.2 schematically depicts the energy changes that occur during the progression of a simple reaction. In order for the reaction to proceed, an activation energy must be overcome in order for the reaction to occur.

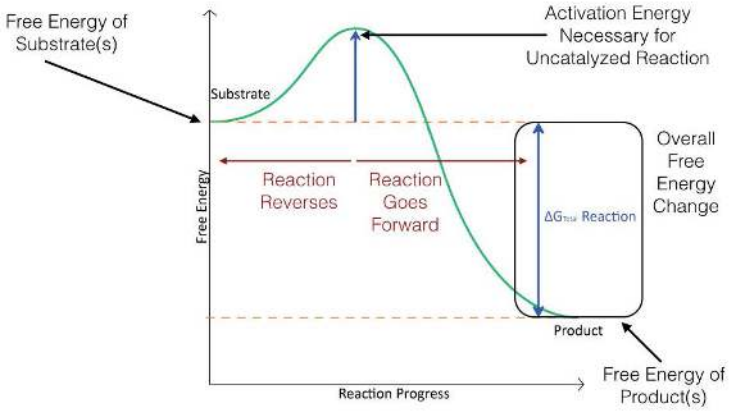


Figure 4.2 - Energy changes during the course of an uncatalyzed reaction. Image by Aleia Kim

In Figure 4.3, the activation energy for a catalyzed reaction is overlaid. As you can see, the reactants start at the same energy level for both catalyzed and uncatalyzed reactions and that the products end at the same energy for both as well. The catalyzed reaction, however, has a lower energy of activation (dotted line) than the uncatalyzed reaction. This is the secret to catalysis - overall ΔG for a reaction does NOT change with catalysis, but the activation energy is lowered.

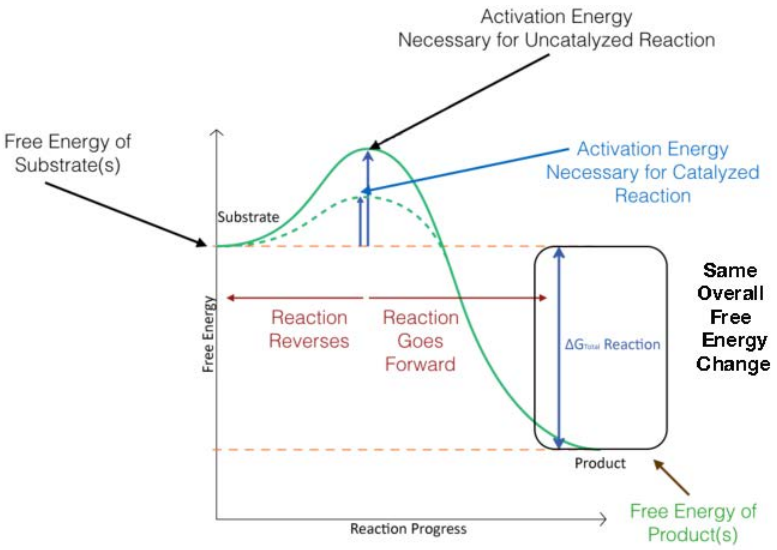


Figure 4.3 - Energy changes during the course of an uncatalyzed reaction (solid green line) and a catalyzed reaction (dotted green line). Image by Aleia Kim

Reversibility

The extent to which reactions will proceed forward is a function of the size of the energy difference between the product and reactant states. The lower the energy of the products compared to the reactants, the larger the percentage of molecules that will be present as products at equilibrium. It is worth noting that since an enzyme lowers the activation energy for a reaction that it can speed the reversal of a reaction just as it speeds a reaction in the forward direction. At equilibrium, of course, no change in concentration of reactants and products occurs. Thus, enzymes speed the time required to reach equilibrium, but do not affect the balance of products and reactants at equilibrium.

Exceptions

The reversibility of enzymatic reactions is an important consideration for equilibrium, the measurement of enzyme kinetics, for Gibbs free energy, for metabolic pathways, and for physiology. There are some minor exceptions to the reversibility of reactions, though. They are related to the disappearance of a substrate or product of a reaction. Consider the first reaction below which is catalyzed by the enzyme carbonic anhydrase:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \tag{3.1.8}$$

In the forward direction, carbonic acid is produced from water and carbon dioxide. It can either remain intact in the solution or ionize to produce bicarbonate ion and a proton. In the reverse direction, water and carbon dioxide are produced. Carbon dioxide, of course, is a gas and can leave the solution and escape.

When reaction molecules are removed, as they would be if carbon dioxide escaped, the reaction is pulled in the direction of the molecule being lost and reversal cannot occur unless the missing molecule is replaced. In the second reaction occurring on the right, carbonic acid (H2CO3) is “removed” by ionization. This too would limit the reaction going back to carbon dioxide in water. This last type of “removal” is what occurs in metabolic pathways. In this case, the product of one reaction (carbonic acid) is the substrate for the next (formation of bicarbonate and a proton).

In the metabolic pathway of glycolysis, ten reactions are connected in this manner and reversing the process is much more complicated than if just one reaction was being considered.

General mechanisms of action

As noted above, enzymatically catalyzed reactions are orders of magnitude faster than uncatalyzed and chemical-catalyzed reactions. The secret of their success lies in a fundamental difference in their mechanisms of action.

Every chemistry student has been taught that a catalyst speeds a reaction without being consumed by it. In other words, the catalyst ends up after a reaction just the way it started so it can catalyze other reactions, as well. Enzymes share this property, but in the middle, during the catalytic action, an enzyme is transiently changed. In fact, it is the ability of an enzyme to change that leads to its incredible efficiency as a catalyst.

Changes

These changes may be subtle electronic ones, more significant covalent modifications, or structural changes arising from the flexibility inherent in enzymes, but not present in chemical catalysts. Flexibility allows movement and movement facilitates alteration of electronic environments necessary for catalysis. Enzymes are, thus, much more efficient than rigid chemical catalysts as a result of their abilities to facilitate the changes necessary to optimize the catalytic process.

Substrate binding

Another important difference between the mechanism of action of an enzyme and a chemical catalyst is that an enzyme has binding sites that not only ‘grab’ the substrate (molecule involved in the reaction being catalyzed), but also place it in a position to be electronically induced to react, either within itself or with another substrate.

The enzyme itself may play a role in the electronic induction or the induction may occur as a result of substrates being placed in very close proximity to each other. Chemical catalysts have no such ability to bind substrates and are dependent upon them colliding in the right orientation at or near their surfaces.

Active site

Reactions in an enzyme are catalyzed at a specific location within it known as the ‘active site’. Substrates bind at the active site and are oriented to provide access for the relevant portion of the molecule to the electronic environment of the enzyme where catalysis occurs.

Enzyme flexibility

As mentioned earlier, a difference between an enzyme and a chemical catalyst is that an enzyme is flexible. Its slight changes in shape (often arising from the binding of the substrate itself) help to optimally position substrates for reaction after they bind.

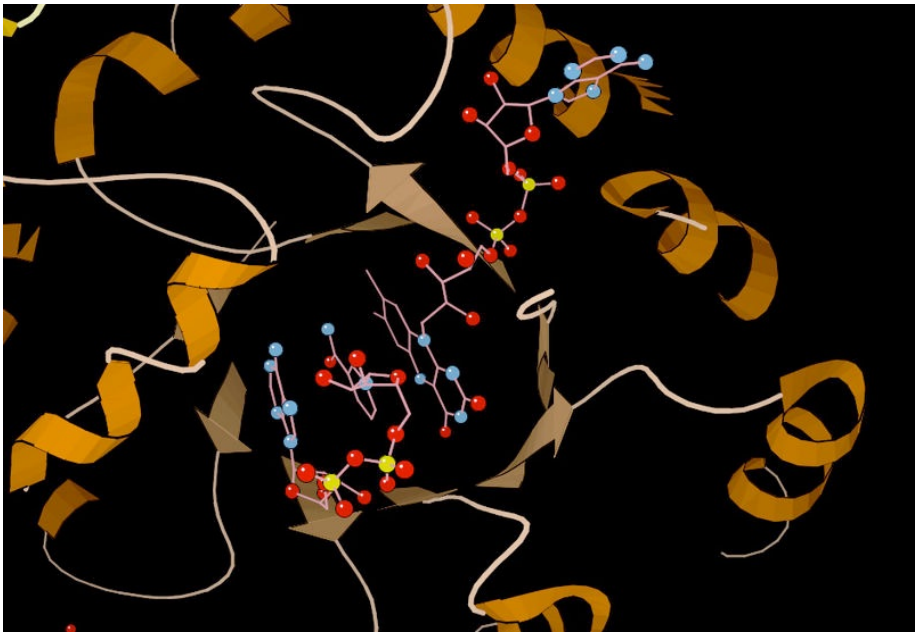


Figure 4.4 - Substrate binding by methylenetetrahydrofolate reductase

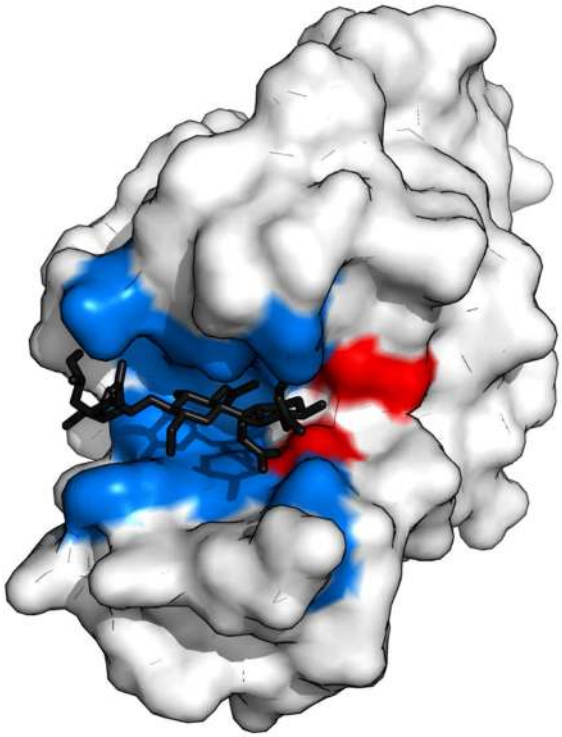


Figure 4.5 - Lysozyme with substrate binding site (blue), active site (red) and bound substrate (black). Wikipedia

Induced fit

These changes in shape are explained, in part, by Koshland's Induced Fit Model of Catalysis (Figure 4.6), which illustrates that not only do enzymes change substrates, but that substrates also transiently change enzyme structure. At the end of the catalysis, the enzyme is returned to its original state. Koshland's model is in contrast to the Fischer Lock and Key model, which says simply that an enzyme has a fixed shape that is perfectly matched for binding its substrate(s). Enzyme flexibility also is important for control of enzyme activity. Enzymes alternate between the T (tight) state, which is a lower activity state and the R (relaxed) state, which has greater activity.

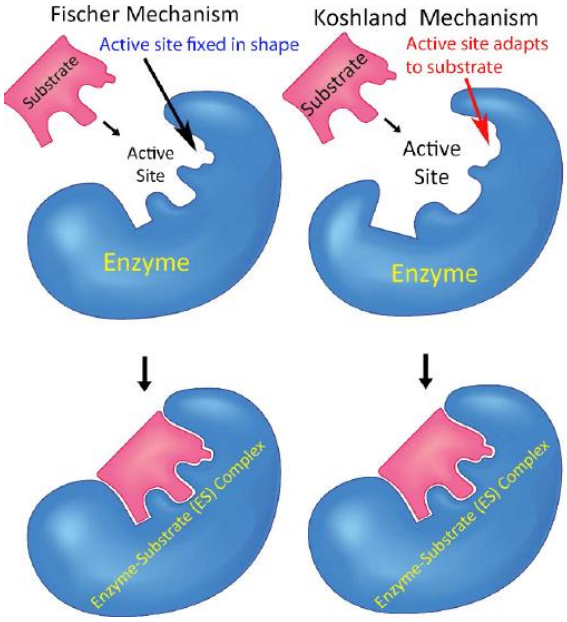


Figure 4.6 - Fischer's lock and key model (left) Vs. Koshland's induced fit model (right). Image by Aleia Kim

Induced Fit

The Koshland Induced Fit model of catalysis postulates that enzymes are flexible and change shape on binding substrate. Changes in shape help to 1) aid binding of additional substrates in reactions involving more than one substrate and/or 2) facilitate formation of an electronic environment in the enzyme that favors catalysis. This model is in contrast to the Fischer Lock and Key Model of catalysis which considers enzymes as having pre-formed substrate binding sites.

Ordered binding

The Koshland model is consistent with multi-substrate binding enzymes that exhibit ordered binding of substrates. For these systems, binding of the first substrate induces structural changes in the enzyme necessary for binding the second substrate.

There is considerable experimental evidence supporting the Koshland model. Hexokinase, for example, is one of many enzymes known to undergo significant structural alteration after binding of substrate. In this case, the two substrates are brought into very close proximity by the induced fit and catalysis is made possible as a result.

Reaction types

Enzymes that catalyze reactions involving more than one substrate, such as

$$A + B \rightleftharpoons C + D \tag{3.1.9}$$

can act in two different ways. Enzymatic reactions can be of several types, as shown in Figure 4.7. In one mechanism, called sequential reactions, at some point in the reaction, both substrates will be bound to the enzyme. There are, in turn, two different ways in which this can occur - random and ordered.

Figure 4.7 - Categories of enzymatic reactions

Types of Reactions	
Single Substrate - Single Product	A ⇄ B
Single Substrate - Multiple Products	A ⇄ B + C
Multiple Substrates - Single Products	A + B ⇄ C
Multiple Substrates - Multiple Products	A + B ⇄ C + D

Consider lactate dehydrogenase, which catalyzes the reaction below:

$$NADH + Pyruvate \rightarrow Lactate + NAD^{+} \tag{3.1.10}$$

This enzyme requires that NADH must bind prior to the binding of pyruvate. As noted earlier, this is consistent with an induced fit model of catalysis. In this case, binding of the NADH changes the enzyme shape/environment so that pyruvate can bind and without binding of NADH, the substrate cannot access the pyruvate binding site. This type of multiple substrate reaction is called **sequential ordered binding**, because the binding of substrates must occur in the right order for the reaction to proceed.

Random binding

A second mechanism of binding/catalysis is exhibited by creatine kinase which catalyzes the following reaction:

$$Creatine + ATP \rightarrow \text{Creatine phosphate} + ADP \tag{3.1.11}$$

For this enzyme, substrates can bind to it in any order. Creatine kinase displays sequential random binding. It is worth mentioning that random binding is not inconsistent with Koshland's induced fit model. Rather, random binding simply means that the enzyme's induced fit doesn't affect substrate binding sites and involves other parts of the enzyme. In summary, sequential binding can occur as ordered binding or as random binding.

Double displacement reactions

Not all enzymes that catalyze multi-substrate reactions, though, bind A and B by the sequential mechanisms above. This other category of enzyme includes those that exhibit what are called “ping-pong” (or double displacement) mechanisms. In these enzymes, the enzyme functions as both a catalyst and a carrier of a group between individually bound substrates. Examples of this type of enzyme include the class of enzymes known as transaminases. A general form of the reactions catalyzed by these enzymes is shown in Figure 4.8.

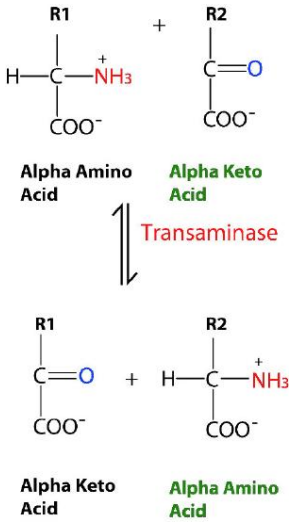


Figure 4.8 - Double displacement reaction mechanism of a transaminase

In reversible transaminase reactions, an oxygen and an amine are swapping between the molecules. It can be represented as follows (where N is the amine and O is the oxygen).

$$A{=}O + C{=}N \rightleftarrows B{-}N + D{=}O$$

This reaction occurs not as one transfer reaction swapping the N and the O, but rather as a set of two half-reactions. In this case, the enzyme is a both donor and a carrier of the group being swapped. The first half-reaction goes as follows

$$A{=}O + Enz{-}N \rightleftarrows B{-}N + Enz{=}O$$

Next a second half-reaction goes as

$$C{-}N + Enz{=}O \rightleftarrows D{=}O + Enz{=}N$$

The sum of these half-reactions then is

$$A{=}O + C{=}N \rightleftarrows B{-}N + D{=}O$$

Note that at no time did the enzyme bind both A and C simultaneously. It is also important to recognize that the enzyme existed in two states - Enz=O and Enz-N. The shuffling of the enzyme between these two states is what gives rise to the ping-pong name of this mechanism - it literally goes back and forth like a ping-pong ball in a table tennis match.

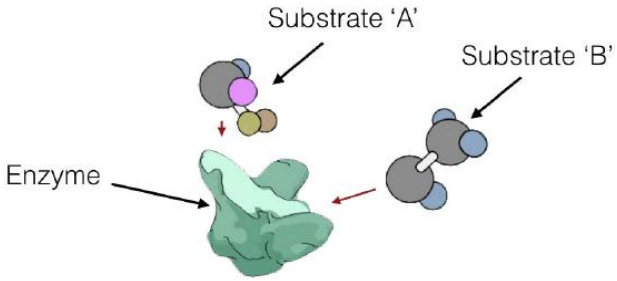


Figure 4.9 - Binding of substrates (A+B) to free enzyme

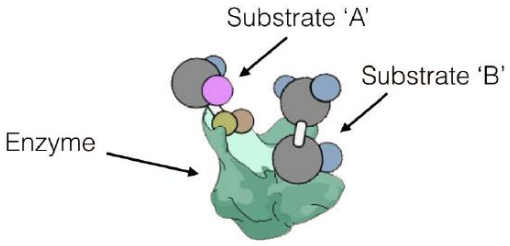


Figure 4.10 ES complex formation

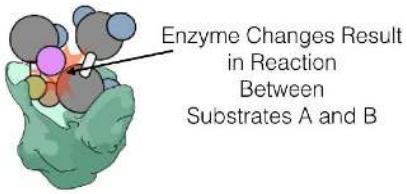


Figure 4.11 - ES* complex - The reaction occurs.

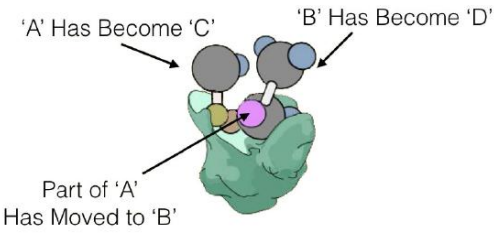


Figure 4.12 - EP complex - the reaction is complete.

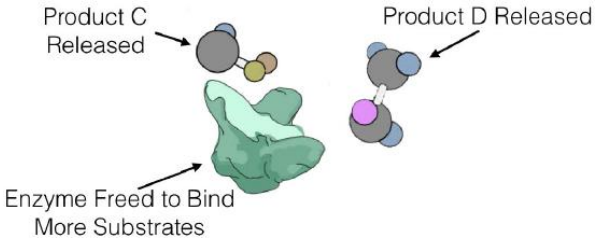


Figure 4.13 - E + P - the products are released

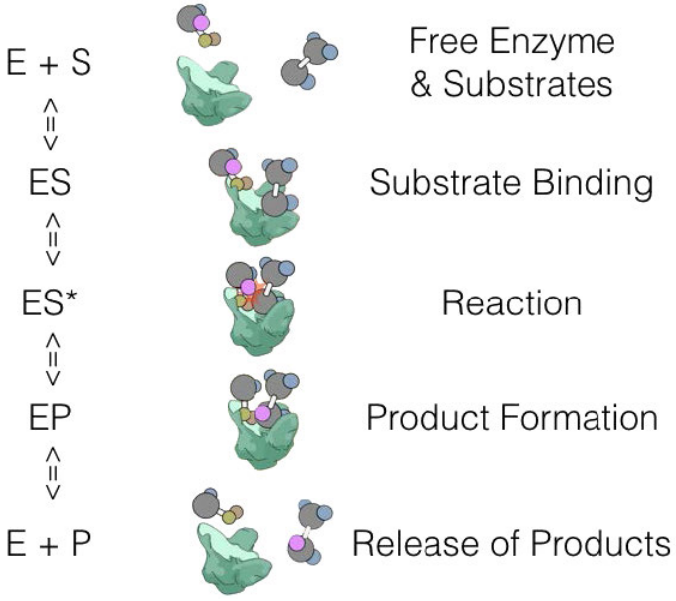


Figure 4.14 - Summary

Enzyme kinetics

To understand how an enzyme enhances the rate of a reaction, we must understand enzyme kinetics. We present a model here proposed by Leonor Michaelis and Maud Menten. In order to understand the model, it is necessary to understand a few parameters.

First, we describe a reaction in simple terms proceeding as follows

E + S ⇄ ES -> E + P

where E is enzyme, S is substrate, and P is product. In this scheme, ES is the Enzyme-Substrate complex, which is simply the enzyme bound to its substrate.

We could define the ES state a bit further with

E + S ⇄ ES -> ES* -> EP -> E + P

where ES* is the activated state and EP is the enzyme-product complex before release of the product.

The first consideration we have is velocity. The velocity of a reaction is the rate of creation of product over time, measured as the concentration of product per time. The time is a critical consideration when measuring velocity. In a closed system (in which an enzyme operates), all reactions will advance towards equilibrium. Enzymatically catalyzed reactions are no different in the end result from non-enzymatic reactions, except that they get to equilibrium faster.

Equilibrium

At equilibrium, the ratio of product to reactant does not change. That is a property of equilibrium. Since the system is closed, the concentration of product over time will not change. The velocity will thus be zero under these conditions and we will have learned nothing about the reaction if we wait too long to study it.

Velocity

Consequently, in Michaelis-Menten kinetics, velocity is measured as initial velocity (Vo). This is accomplished by measuring the rate of formation of product early in the reaction before equilibrium is established and under these conditions, there is very little if any of the reverse reaction occurring.

The other two assumptions are related. First, we use conditions where there is much more substrate than enzyme. This makes sense. If the substrate is not in great excess, then the enzyme's conversion of substrate to product will occur much faster than the enzyme can bind substrate.

Waiting for substrate

Thus, the enzyme would "wait" for substrate to bind if there were not sufficient amounts of it to bind to the enzyme in a timely fashion (when substrate concentration is low). This would not give an accurate measure of velocity, since the enzyme would be inactive a good deal of the time. Because of this, we assume saturation of the enzyme with substrate will give a maximal velocity of the reaction.

Steady state

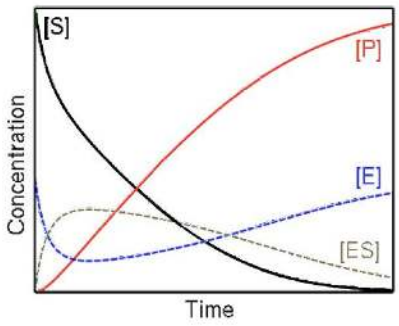


Figure 4.15 - Concentration of product (P), substrate (S), enzyme (E), and enzyme-substrate complex (ES) versus time for an enzymatic reaction

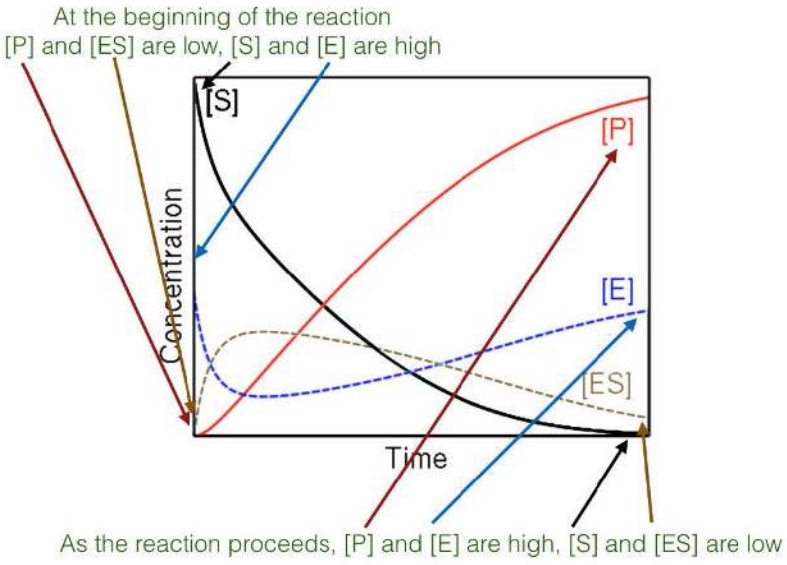


Figure 4.16 - Change in concentration of reaction materials over time

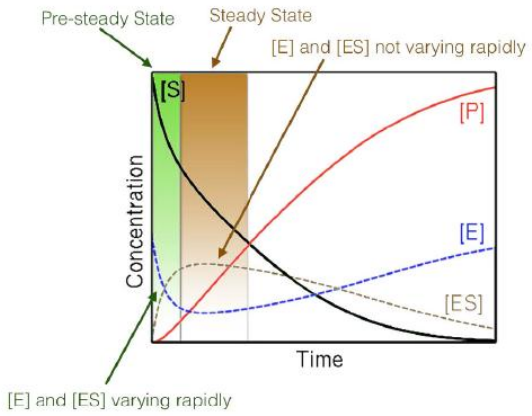


Figure 4.17 - Steady state versus non-steady state conditions

Last, the high concentration of substrate combined with measuring initial conditions results in studying reactions that are under so-called steady state conditions (Figure 4.17). When steady state occurs, the concentration of the ES complex over time is not significantly changing during the period of analysis.

Reiterating, the three assumptions for Michaelis-Menten kinetics are

1. Measurement of initial velocity of a reaction
2. Substrate in great excess compared to enzyme
3. Reaction conditions occurring under steady state

Experimental considerations

Now we turn our attention to how studies of the kinetic properties of an enzyme are conducted. To perform an analysis, one would do the following experiment - 20 different tubes would be set up with enzyme buffer (to keep the enzyme stable), the same amount of enzyme, and then a different amount of substrate in each tube, ranging from tiny amounts in the first tubes to very large amounts in the last tubes. The reaction would be allowed to proceed for a fixed, short amount of time and then the reaction would be stopped and the amount of product contained in each tube would be determined.

The initial velocity (V0) of the reaction then would be the concentration of product found in each tube divided by the time that the reaction was allowed to run. Data from the experiment would be plotted on a graph using initial velocity (V0) on the Y-axis and the concentration of substrate on the X-axis, each tube, of course having a unique reaction velocity corresponding to a unique substrate concentration.

For an enzyme following Michaelis-Menten kinetics, a curve like that shown in Figure 4.18 or 4.19 would result. At low concentration of substrate, it is limiting and the enzyme converts it into product as soon as it can bind it. Consequently, at low concentrations of substrate, the rate of increase of [P] is almost linear with [S] (Figure 4.19).

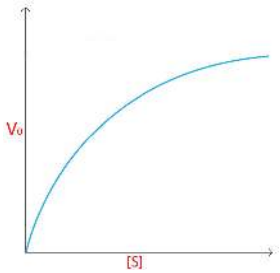


Figure 4.18 - Kinetics of an enzyme obeying Michaelis-Menten kinetics. Image by Aleia Kim

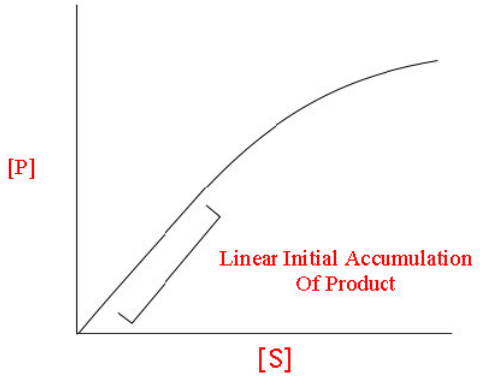


Figure 4.19 - Linear relationship between [P] and [S] at low [S]

Non-linear increase

As the substrate concentration increases, however, the velocity of the reaction in tubes with higher substrate concentration ceases to increase linearly and instead begins to flatten out, indicating that as the substrate concentration gets higher and higher, the enzyme has a harder time keeping up to convert the substrate to product.

Saturation

Not surprisingly, when the enzyme becomes completely saturated with substrate, it will not have to wait for substrate to diffuse to it and will therefore be operating at maximum velocity.

For an enzyme following Michaelis-Menten kinetics will have its velocity (v) at any given substrate concentration given by the following equation:

$$v = \frac{V_{\max}[S]}{K_m + [S]}$$

V_{max}

Two terms in the equation above require explanation. The first is V_{max}. It refers to the maximum velocity of an enzymatic reaction. Maximum velocity for a reaction occurs when an enzyme is saturated with substrate. Saturation is important because it means (per the assumption above) that none of the enzyme molecules are “waiting” for substrate after a product is released. Saturation ensures that another substrate is always instantly available. The unit of V_{max} is concentration of product per time = [P]/time.

On a plot of initial velocity versus substrate concentration (V0 vs. [S]), V_{max} is the value on the Y axis that the curve asymptotically approaches (dotted line in Figure 4.20). It should be noted that the value of V_{max} depends on the amount of enzyme used in a reaction. If you double the amount of enzyme used, you will double the V_{max}. If one wanted to compare the velocities of two different enzymes, it would be necessary to use the same amounts of enzyme in the reaction each one catalyzes.

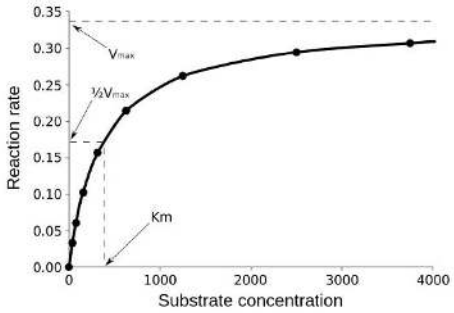


Figure 4.20 - V_{max}, $V_{max}/2$, and Km

Km

The second term is Km (also known as Ks). Referred to as the Michaelis constant, Km is the substrate concentration that causes the enzyme to work at half of maximum velocity (Vmax/2). What it measures, in simple terms, is the affinity an enzyme has for its substrate. The value of Km is inversely related to the affinity of the enzyme for its substrate. Enzymes with a high Km value will have a lower affinity for their substrate (will take more substrate to get to Vmax/2) whereas those with a low Km will have high affinity and take less substrate to get to Vmax/2. The unit of Km is concentration.

Affinities of enzymes for substrates vary considerably, so knowing Km helps us to understand how well an enzyme is suited to the substrate being used. Measurement of Km depends on the measurement of Vmax.

Common mistake

A common mistake students make in describing Vmax is saying that Km = Vmax /2. This is, of course, not true. Km is a substrate concentration and is the amount of substrate it takes for an enzyme to reach Vmax /2. On the other hand Vmax /2 is a velocity and a velocity certainly cannot equal a concentration.

Kcat

It is desirable to have a measure of velocity that is independent of enzyme concentration. Remember that Vmax depended on the amount of enzyme used. For this, we use the Kcat, also known as the turnover number. Kcat is a number that requires one to first determine Vmax for an enzyme and then divide the Vmax by the concentration of enzyme used to determine Vmax. Thus,

Kcat = Vmax /[Enzyme]

Since Vmax has units of concentration per time and [Enzyme] has units of concentration, the units on Kcat are time-1. While that might seem unintuitive, it means that the value of Kcat is the number of molecules of product made by each molecule of enzyme in the time given. So, a Kcat value of 1000/sec means each enzyme molecule in the reaction at Vmax is producing 1000 molecules of product per second. Note that since Kcat is a calculated value, it cannot be read from a V vs [S] graph as Vmax and Km can.

Enzyme	Turnover Number (per second)
Carbonic anhydrase	600,000
3-Ketoesteroid isomerase	280,000
Acetylcholinesterase	25,000
Penicillinase	2,000
Lactate dehydrogenase	1,000
Chymostrypsin	100
DNA Polymerase I	15
Tryptophan synthetase	2
Lysozyme	0.5

Figure 4.21 - Kcat (turnover number) values for several enzymes. Image by Aleia Kim

Amazing Kcat values

A Kcat value of 1000 molecules of product per enzyme per second might seem like a high value, but there are enzymes known (carbonic anhydrase, for example) that have a Kcat value of over 600,000/second (Figure 4.21). This astonishing value illustrates clearly why enzymes seem almost magical in their action. In contrast to V_{max}, which varies with the amount of enzyme used, Kcat is a constant for an enzyme under given conditions.

As seen earlier, enzymes that follow Michaelis-Menten kinetics produce hyperbolic plots of Velocity (V0) versus Substrate Concentration [S] (Figure 4.18). Not all enzymes, though, follow Michaelis-Menten kinetics. Many enzymes have multiple protein

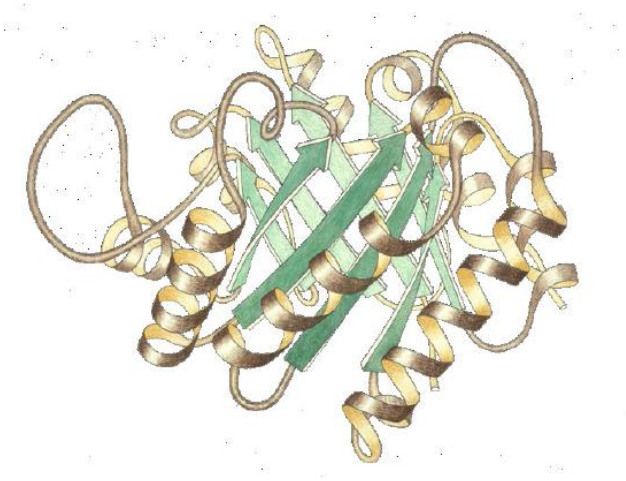
subunits and these sometimes interact differently upon binding of a substrate or an external molecule. See ATCase (HERE) for an example.

Perfect enzymes

Now, if we think about what an ideal enzyme might be, it would be one that has a very high velocity and a very high affinity for its substrate. That is, it wouldn't take much substrate to get to Vmax/2 and the Kcat would be very high. Such enzymes would have values of Kcat / Km that are maximum. Interestingly, there are several enzymes that have this property and their maximal Kcat / Km values are all approximately the same. Such enzymes are referred to as being "perfect" because they have reached the maximum possible value.

Enzyme	K_{Cat} / K_M ($s^{-1}M^{-1}$)
Acetylcholinesterase	1.6×10^8
Carbonic anhydrase	8.3×10^7
Catalase	4.0×10^7
Crotonase	2.8×10^8
Fumarase	1.6×10^8
Triose phosphate isomerase	2.4×10^8
β-Lactamase	1.0×10^8
Superoxide dismutase	7.0×10^9

Figure 4.22 - Kcat/Km values for perfect enzymes. Image by Aleia Kim



Wikipedia

Figure 4.23 - Triose phosphate isomerase - A perfect enzyme.

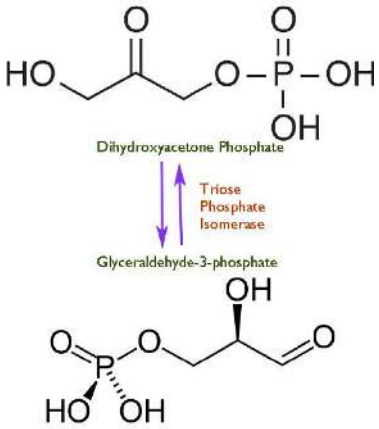


Figure 4.24 - Triose phosphate isomerase-catalyzed reaction

Diffusion limitation

Why should there be a maximum possible value of Kcat / Km? The answer is that movement of substrate to the enzyme becomes the limiting factor for perfect enzymes. Movement of substrate by diffusion in water has a fixed rate at any temperature and that limitation ultimately determines the maximum speed an enzyme can catalyze at. In a macroscopic world analogy, factories can't make products faster than suppliers can deliver materials. It is safe to say for a perfect enzyme that the only speed limit it has is the rate of substrate diffusion in water.

Given the "magic" of enzymes alluded to earlier, it might seem that all enzymes should have evolved to be "perfect." There are very good reasons why most of them have not.

Speed

Speed is a dangerous thing. The faster a reaction proceeds in catalysis by an enzyme, the harder it is to control. As we all know from learning to drive, speeding causes accidents. Just as drivers need to have speed limits for operating automobiles, so too must cells exert some control on the 'throttle' of their enzymes. In view of this, one might wonder then why any cells have evolved any enzymes to perfection. There is no single answer to the question, but a common one is illustrated by triose phosphate isomerase, which catalyzes a reaction in glycolysis shown in Figure 4.24.

The enzyme appears to have evolved this ability because at lower velocities, there is breakdown of an unstable enediol intermediate that then readily forms methyl glyoxal, a cytotoxic compound (Figure 4.25). Speeding up the reaction provides less opportunity for the unstable intermediate to accumulate and fewer undesirable byproducts to be made.

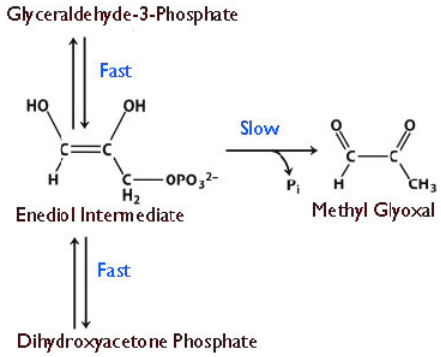


Figure 4.25 - A high speed reaction avoids production of methyl glyoxal. Image by Aleia Kim

Dissociation constant

In studying proteins and ligands, it is important to understand the "tightness" with which a protein (P) "holds onto" a ligand (L). This is measured with the dissociation constant (K_d). The formation of a ligand-protein complex LP occurs as

$$L + P \rightleftharpoons LP \tag{3.1.12}$$

The dissociation of the complex, therefore, is the reverse of this reaction, or

$$LP \rightleftharpoons L + P \tag{3.1.13}$$

so the corresponding dissociation constant is defined as

$$K_d = \frac{[L][P]}{[LP]} \tag{3.1.14}$$

where $[L]$, $[P]$, and $[LP]$ are the molar concentrations of the protein, ligand and the complex when they are joined together.

Smaller values of K_d indicate tight binding, whereas larger values indicate loose binding. The dissociation constant is the inverse of the association constant.

$$K_a = \frac{1}{K_d} \tag{3.1.15}$$

Where multiple molecules bond together, such as

$$J_xK_y \rightleftharpoons xJ + yK \tag{3.1.16}$$

The complex J_xK_y is breaking down into x subunits of J and y subunits of K. The dissociation constant is then defined as

$$K_d = \frac{[J]^x[K]^y}{[J_xK_y]} \tag{3.1.17}$$

where $[J]$, $[K]$, and $[J_xK_y]$ are the concentrations of J, K, and the complex J_xK_y, respectively.

Lineweaver-Burk plots

The study of enzyme kinetics is typically the most math intensive component of biochemistry and one of the most daunting aspects of the subject for many students. Although attempts are made to simplify the mathematical considerations, sometimes they only serve to confuse or frustrate students. Such is the case with modified enzyme plots, such as Lineweaver-Burk (Figure 4.26).

Indeed, when presented by professors as simply another thing to memorize, who can blame students? In reality, both of these plots are aimed at simplifying the determination of parameters, such as K_m and V_{max}. In making either of these modified plots, it is important to recognize that the same data is used as in making a V0 vs. [S] plot. The data are simply manipulated to make the plotting easier.

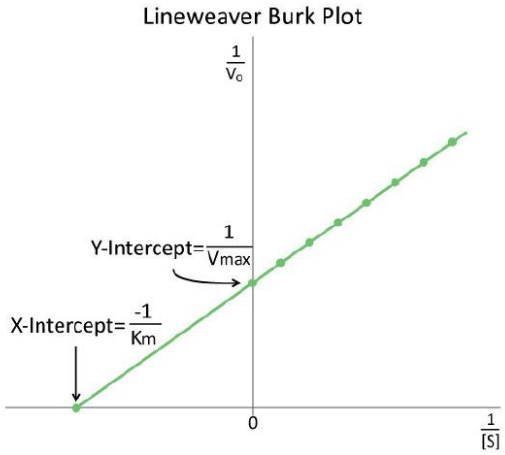


Figure 4.26 - A Lineweaver-Burk plot of $1/V_0$ vs $1/[S]$. Image by Aleia Kim

Double reciprocal

For a LineWeaver-Burk plot, the manipulation is using the reciprocal of the values of both the velocity and the substrate concentration. The inverted values are then plotted on a graph as 1/V0 vs. 1/[S]. Because of these inversions, Lineweaver-Burk

plots are commonly referred to as ‘double-reciprocal’ plots. As can be seen in Figure 4.26, the value of Km on a Lineweaver Burk plot is easily determined as the negative reciprocal of the x-intercept , whereas the Vmax is the inverse of the y-intercept. Other related manipulation of kinetic data include Eadie-Hofstee diagrams, which plots V0 vs V0/[S] and gives Vmax as the Y-axis intercept with the slope of the line being -Km.

Molecularity of reactions

The term molecularity refers to the number of molecules that must come together in order for a reaction to take place. Reactions of the sort of A -> B (where ‘A’ is the reactant and ‘B’ is the product) are unimolecular, since A is directly changed into B. The rate of the reaction is related only to the concentration of reactant A. For a bimolecular reaction where A + B ⇄ C the reaction depends on the concentration of both A and B and its rate will be related to the product of the concentration of A and of B.

Coenzymes

Organic molecules that assist enzymes and facilitate catalysis are co-factors called coenzymes. The term co-factor is a broad category usually subdivided into inorganic ions and coenzymes. If the coenzyme is very tightly or covalently bound to the enzyme, it is referred to as a prosthetic group. Enzymes without their co-factors are inactive and referred to as apoenzymes. Enzymes containing all of their co-factors are called holoenzymes.

Cofactor	Enzyme
Coenzyme	
5′-Deoxyadenosyl cobalamin	Methylmalonyl mutase
Biotin	Pyruvate carboxylase
Coenzyme A (CoA)	Acetyl CoA carboxylase
Flavin adenine nucleotide	Monoamine oxidase
Nicotinamide adenine dinucleotide	Lactate dehydrogenase
Pyridoxal phosphate	Glycogen phosphorylase
Tetrahydrofolate	Thymidylate synthase
Thiamine pyrophosphate	Pyruvate dehydrogenase
Metal	
K^+	Propionyl CoA carboxylase
Mg^{2+}	Restriction Endonucleases; Hexokinase
Mn	Superoxide dismutase
Mo	Nitrate reductase
Ni^{2+}	Urease
Se	Glutathione peroxidase
Zn^{2+}	Carbonic anhydrase; Carboxypeptidase

Figure 4.27 - Enzyme cofactors. Image by Aleia Kim

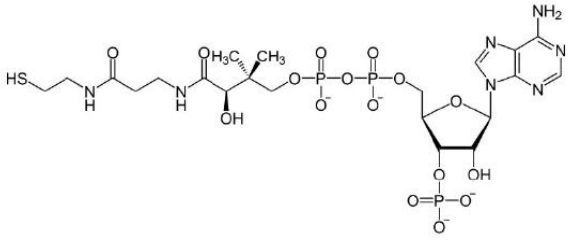


Figure 4.28 - Coenzyme A (CoA)

Pre-steady state kinetic studies

In the study of kinetic rates of enzymatic reactions, time zero is a very critical point. It establishes when the mixing of substrate with enzyme and measurement of formation of product begins. At time zero, there is no product. As shown in Figure 4.29, the appearance of product (on a short time scale) goes through an early burst phase with a steep slope for [product]/time and then changes.

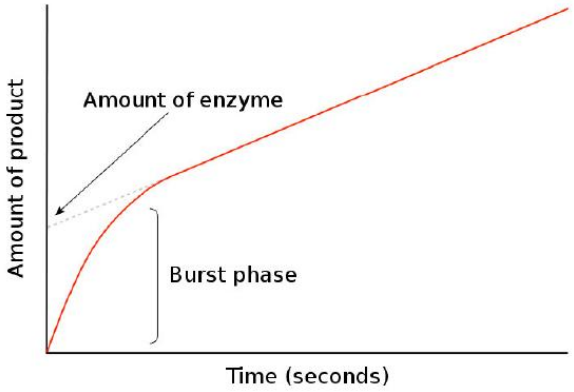


Figure 4.29 - Burst phase of product formation

This change occurs during a critical period in an enzymatic reaction and gives information about the rate of reaction cycles. The duration of the burst phase tells how long a single reaction turnover occurs, whereas the slow of the line post-burst phase tells the amount of "functional" enzyme performing the reaction.

After the burst phase, the slope of the line of the amount of product versus time decreases. This is due to the reaction entering conditions of steady state, used to study Michaelis-Menten kinetics. In steady state conditions, the amount of the enzyme-substrate complex (ES) is relatively constant over time. In simple terms, this occurs when the rate of formation of the ES complex equals the rate of conversion of the substrate to product by the enzyme with release of the product.

Earlier events

Events occurring prior to the conditions of steady state are referred to as pre-steady state. Depending on the enzyme, in as short as a few milliseconds, steady state conditions can be present meaning that if one hopes to study formation of reaction intermediates in pre-steady state, tools for this analysis must work very rapidly. One instrument commonly used for studying pre-steady state kinetics is called a stopped flow instrument.

It loads an enzyme solution and a substrate into separate syringes whose output is pointed into a mixing chamber. The solutions are rapidly mixed and measurements of product concentration begin. With a stopped flow instrument, dead times (time between mixing and detection) can be achieved of as small as 0.3 msec.

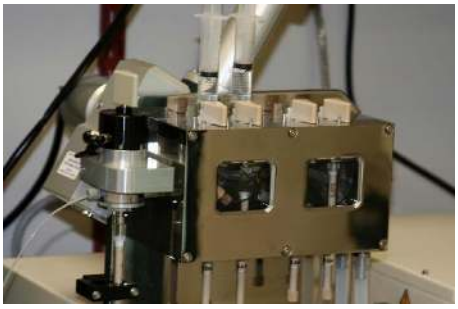
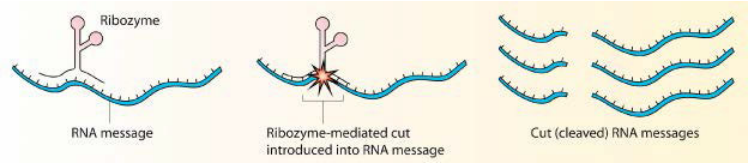


Figure 4.30 - Stopped flow instrument for studying pre-steady state kinetics. Wikipedia

Ribozymes

Proteins do not have a monopoly on acting as biological catalysts. Some RNA molecules are also capable of speeding reactions. The most famous of these molecules was discovered by Tom Cech in the early 1980s Studying excision of an intron in Tetrahymena, Cech was puzzled at his inability to find any proteins catalyzing the process. Ultimately, the catalysis was recognized as coming from the intron itself. It was a self-splicing RNA and since then, many other examples of catalytic RNAs have been found. Catalytic RNA molecules are known as ribozymes.



359

Figure 4.31 - Cleavage of an RNA by a ribozyme. Wikipedia

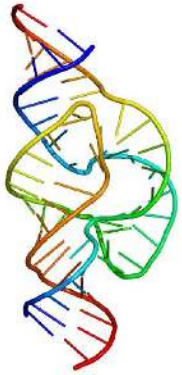


Figure 4.32 - Hammerhead ribozyme. Wikipedia

Not unusual

Ribozymes, however, are not rarities of nature. The protein-making ribosomes of cells are essentially giant ribozymes. The 23S rRNA of the prokaryotic ribosome and the 28S rRNA of the eukaryotic ribosome catalyze the formation of peptide bonds.

Ribozymes are also important in our understanding of the evolution of life on Earth. They have been shown to be capable via selection to evolve self-replication. Indeed, ribozymes actually answer a chicken/egg dilemma - which came first, enzymes that do the work of the cell or nucleic acids that carry the information required to produce the enzymes. As both carriers of genetic information and catalysts, ribozymes are likely both the chicken and the egg in the origin of life.

This page titled 3.1: Basic Principles of Catalysis is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **4.1: Basic Principles of Catalysis** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

3.2: Control of Enzymatic Activity

A printable version of this section is here: BiochemFFA_4_2.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

Regulation of enzyme activity

Apart from their ability to greatly speed the rates of chemical reactions in cells, enzymes have another property that makes them valuable. This property is that their activity can be regulated, allowing them to be activated and inactivated, as necessary. This is tremendously important in maintaining homeostasis, permitting cells to respond in controlled ways to changes in both internal and external conditions.

Inhibition of specific enzymes by drugs can also be medically useful. Understanding the mechanisms that control enzyme activity is, therefore, of considerable importance.

Inhibition

We will first discuss four types of enzyme inhibition – competitive, non-competitive, uncompetitive, and suicide inhibition. Of these, the first three types are reversible. The last one, suicide inhibition, is not.

Competitive inhibition

Probably the easiest type of enzyme inhibition to understand is competitive inhibition and it is the one most commonly exploited pharmaceutically. Molecules that are competitive inhibitors of enzymes resemble one of the normal substrates of an enzyme. An example is methotrexate, which resembles the folate substrate of the enzyme dihydrofolate reductase (DHFR). This enzyme normally catalyzes the reduction of folate, an important reaction in the metabolism of nucleotides.

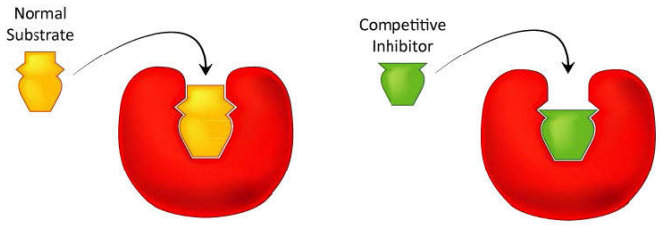


Figure 4.33 - Competitive inhibitors resemble the normal substrate and compete for binding at the active site. Image by Aleia Kim

Inhibitor binding

When the drug methotrexate is present, some of the DHFR enzyme binds to it, instead of to folate, and during the time methotrexate is bound, the enzyme is inactive and unable to bind folate. Thus, the enzyme is inhibited. Notably, the binding site on DHFR for methotrexate is the active site, the same place that folate would normally bind. As a result, methotrexate 'competes' with folate for binding to the enzyme. The more methotrexate there is, the more effectively it competes with folate for the enzyme's active site. Conversely, the more folate there is, the less of an effect methotrexate has on the enzyme because folate outcompetes it.

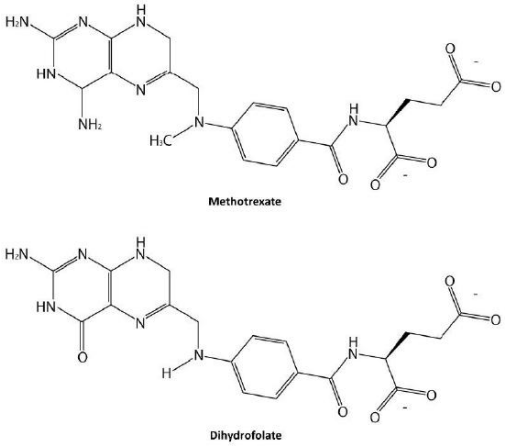


Figure 4.34 - Methotrexate and dihydrofolate. Image by Ben Carson

No effect on Vmax

How do we study competitive inhibition? It is typically done as follows. First, one performs a set of V0 vs. [S] reactions without inhibitor (20 or so tubes, with buffer and constant amounts of enzyme, varying amounts of substrate, equal reaction times). V0 vs. [S] is plotted (Figure 4.35 red line), as well as 1/V0 vs. 1/[S] (Figure 4.36 green line). Next, a second set of reactions is performed in the same manner as before, except that a fixed amount of the methotrexate inhibitor is added to each tube. At low concentrations of substrate, the methotrexate competes for the enzyme effectively, but at high concentrations of substrate, the inhibitor will have a much reduced effect, since the substrate outcompetes it, due to its higher concentration (remember that the inhibitor is at fixed concentration).

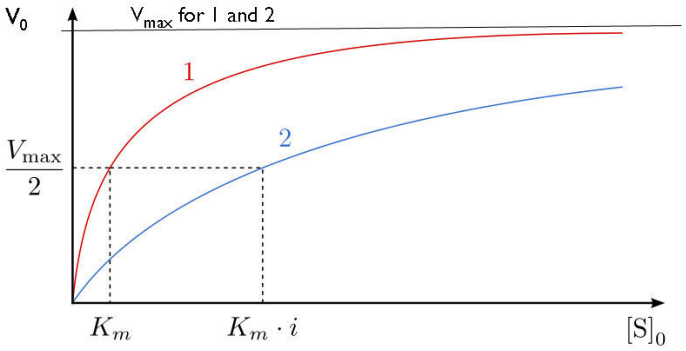


Figure 4.35 V0 vs [S] Plots for uninhibited reactions (red) and competitively inhibited reactions (blue). Both ultimately have same Vmax

Graphically, the results of these inhibitor experiments are shown in Figure 4.35 (blue line) and Figure 4.36 (orange line). Notice that at high substrate concentrations, the competitive inhibitor has essentially no effect, causing the V_{max} for the enzyme to remain unchanged. To reiterate, this is due to the fact that at high substrate concentrations, the inhibitor doesn't compete well. However, at lower substrate concentrations, it does.

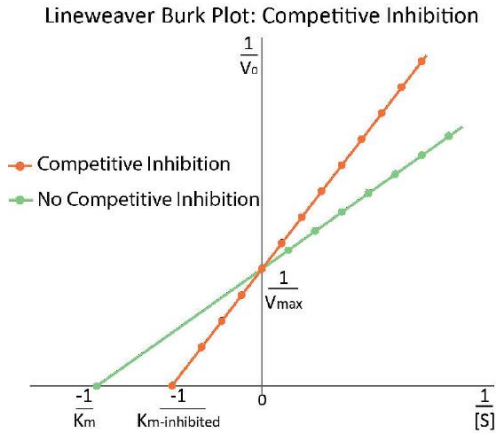


Figure 4.36 - Lineweaver-Burk plots - uninhibited reactions (green). Competitively inhibited reactions (orange). Lines cross on Y-axis at 1/Vmax Since Vmax is the same for both reactions. Image by Aleia Kim

Increased K_m

In competitively inhibited reactions, the apparent Km of the enzyme for the substrate increases ($-1/K_m$ gets closer to zero - red line in Figure 4.36) when the inhibitor is present compared to when the inhibitor is absent, thus illustrating the better competition of the inhibitor at lower substrate concentrations. It may not be obvious why we call the changed Km the apparent Km of the enzyme. The reason is that the inhibitor doesn't actually change the enzyme's affinity for the folate substrate. It only appears to do so. This is because of the way that competitive inhibition works. When the competitive inhibitor binds the enzyme, it is effectively 'taken out of action.' Inactive enzymes have NO affinity for substrate and no activity either. We can't measure Km for an inactive enzyme.

The enzyme molecules that are not bound by methotrexate can, in fact, bind folate and are active. Methotrexate has no effect on them and their Km values are unchanged. Why then, does Km appear higher in the presence of a competitive inhibitor? The reason is that the competitive inhibitor is having a greater effect of reducing the amount of active enzyme at lower concentrations of substrate than it does at higher concentrations of substrate. When the amount of enzyme is reduced, one must have more substrate to supply the reduced amount of enzyme sufficiently to get to Vmax/2.

It is worth noting that in competitive inhibition, the percentage of inactive enzyme changes drastically over the range of [S] values used. To start, at low [S] values, the greatest percentage of the enzyme is inhibited. At high [S], no significant percentage of enzyme is inhibited. This is not always the case, as we shall see in non-competitive inhibition.

Non-competitive inhibition

A second type of inhibition employs inhibitors that do not resemble the substrate and bind not to the active site, but rather to a separate site on the enzyme (Figure 4.37). The effect of binding a non-competitive inhibitor is significantly different from binding a competitive inhibitor because there is no competition. In the case of competitive inhibition, the effect of the inhibitor could be reduced and eventually overwhelmed with increasing amounts of substrate. This was because increasing substrate made increasing percentages of the enzyme active. With non-competitive inhibition, increasing the amount of substrate has no effect on the percentage of enzyme that is active. Indeed, in non-competitive inhibition, the percentage of enzyme inhibited remains the same through all ranges of [S].

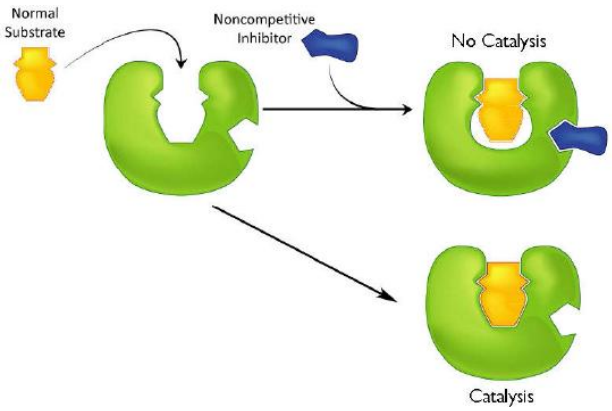


Figure 4.37 - Non-competitive inhibition - inhibitor does not resemble the substrate and binds to a site other than the active site. Image by Aleia Kim

This means, then, that non-competitive inhibition effectively reduces the amount of enzyme by the same fixed amount in a typical experiment at every substrate concentration used The effect of this inhibition is shown in Figure 4.38 & 4.39. As you can see, V_{max} is reduced in non-competitive inhibition compared to uninhibited reactions.

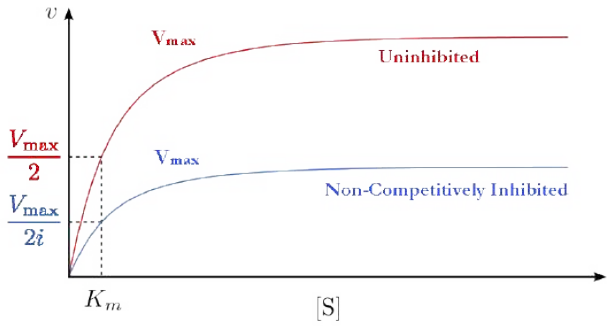


Figure 4.38 - V0 vs [S] plots of uninhibited reactions (red) and non-competitively inhibited reactions (blue). Vmax is reduced, but Km values are unchanged in non-competitively inhibited reactions

This makes sense if we remember that Vmax is dependent on the amount of enzyme present. Reducing the amount of enzyme present reduces V_{max}. In competitive inhibition, this doesn't occur detectably, because at high substrate concentrations, there is essentially 100% of the enzyme active and the V_{max} appears not to change. Additionally, Km for non-competitively inhibited reactions does not change from that of uninhibited reactions. This is because, as noted previously, one can only measure the K_m of active enzymes and K_m is a constant for a given enzyme.

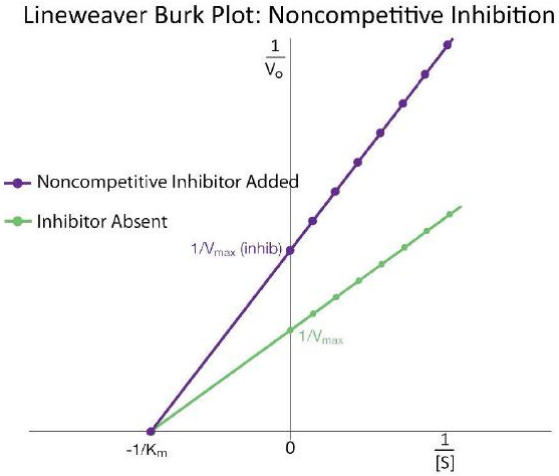


Figure 4.39 - Lineweaver Burk plots of uninhibited reactions (green) and non-competitively inhibited reactions (purple). Image by Aleia Kim

Uncompetitive inhibition

A third type of enzymatic inhibition is that of uncompetitive inhibition, which has the odd property of a reduced Vmax as well as a reduced Km. The explanation for these seemingly odd results is rooted in the fact that the uncompetitive inhibitor binds only to the enzyme-substrate (ES) complex (Figure 4.40). The inhibitor-bound complex forms mostly under concentrations of high substrate and the ES-I complex cannot release product while the inhibitor is bound, thus explaining the reduced V_{max}.

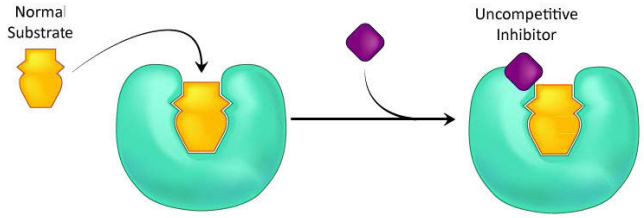


Figure 4.40 - Uncompetitive inhibition. Image by Aleia Kim

The reduced Km is a bit harder to conceptualize. The reason is that the inhibitor-bound complex effectively reduces the concentration of the ES complex. By Le Chatelier's Principle, a shift occurs to form additional ES complex, resulting in less free enzyme and more enzyme in the forms ES and ESI (ES with inhibitor). Decreases in free enzyme correspond to an enzyme with greater affinity for its substrate. Thus, paradoxically, uncompetitive inhibition both decreases V_{max} and increases an enzyme's affinity for its substrate (K_m - Figures 4.41 & 4.42).

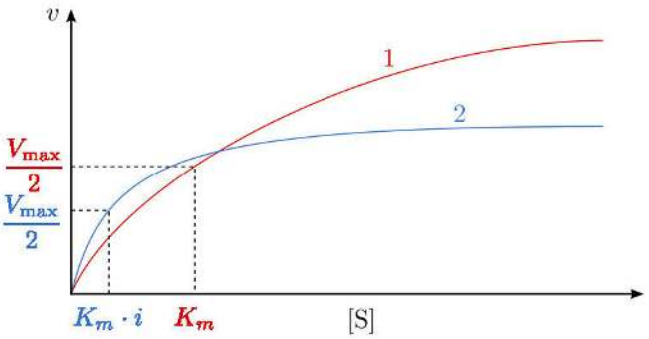


Figure 4.41 - V0 vs [S] plot for uncompetitive inhibition (blue) and uninhibited reactions (red)

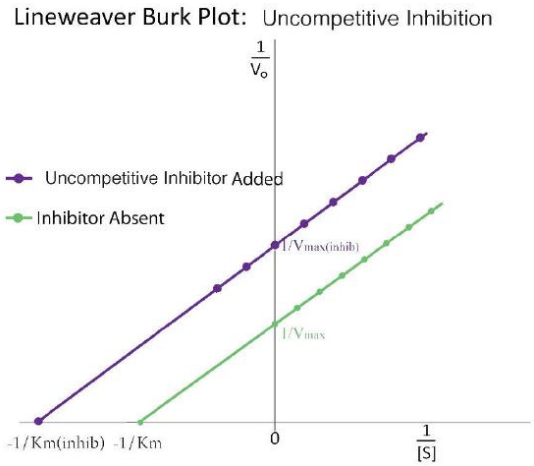


Figure 4.42 - Uncompetitive inhibition (purple) and uninhibited reactions (green). Image by Aleia Kim

Irreversible (Suicide) inhibition

In contrast to the first three types of inhibition, which involve reversible binding of the inhibitor to the enzyme, suicide inhibition is irreversible, because the inhibitor becomes covalently bound to the enzyme during the inhibition. Suicide inhibition rather closely

resembles competitive inhibition because the inhibitor generally resembles the substrate and binds to the active site of the enzyme. The primary difference is that the suicide inhibitor is chemically reactive in the active site and makes a bond with it that precludes its removal. Such a mechanism is that employed by penicillin (Figure 4.43), which covalently links to the bacterial enzyme, DD transpeptidase and stops it from functioning. Since the normal function of the enzyme is to make a bond necessary for the peptidoglycan complex of the bacterial cell wall, the cell wall cannot properly form and bacteria cannot reproduce.

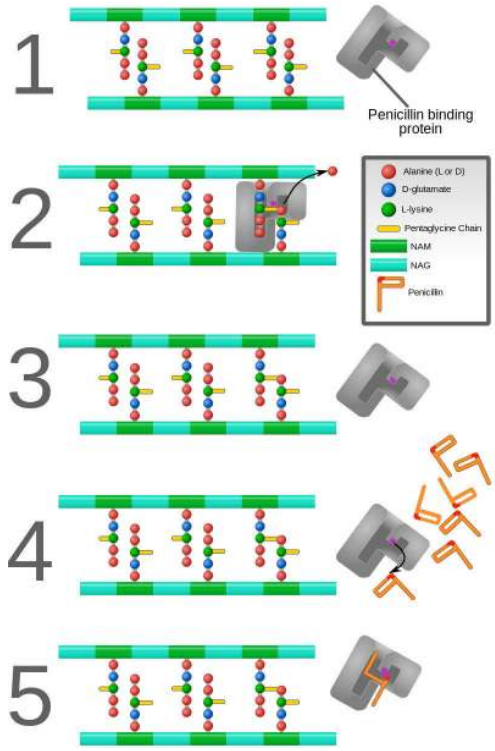


Figure 4.43 - Action of penicillin. DD-transpeptidase builds peptidoglycan layer of bacterial cell wall (1-3). Binding of penicillin by DD-transpeptidase stops peptidoglycan synthesis (4-5). Wikipedia

Control of enzymes

It is appropriate to talk at this point about mechanisms cells use to control enzymes. There are four general methods that are employed:

1. allosteric regulation,
2. covalent modification,
3. access to substrate, and
4. control of enzyme level via synthesis or breakdown.

Some enzymes are controlled by more than one of these methods.

Allosteric regulation

The term allosteric regulation, or allostersim, refers to the fact that the activity of certain enzymes can be affected by the binding of small molecules. Molecules causing allosteric effects come in two classifications. A substrate for an enzyme can also regulate that same enzyme. These allosteric regulators are generally called **homotropic effectors**. Regulators that are not substrates are called **heterotropic effectors.**

The homotropic effectors usually are activators of the enzymes they bind to and the results of their action can be seen in the conversion of the hyperbolic curve typical of a V0 vs. [S] plot for an enzyme (Figure 4.18), being converted to a sigmoidal plot (Figure 4.44). This is due to the conversion of the enzyme from the T-state to the R-state on binding the substrate/homotropic effector.

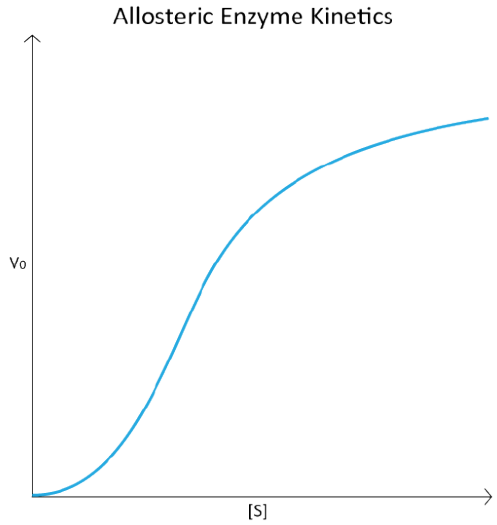


Figure 4.44 - Kinetic profile of an allosteric enzyme whose activity is controlled by a homotropic effector. Image by Aleia Kim

The V0 vs. [S] plot of allosteric enzyme reactions resembles the oxygen binding curve of hemoglobin (see Figure 2.83). Even though hemoglobin is not an enzyme and is thus not catalyzing a reaction, the similarity of the plots is not coincidental. In both cases, the binding of an external molecule is being measured – directly, in the hemoglobin plot, and indirectly by the V0 vs. [S] plot, since substrate binding is a factor in enzyme reaction velocity.

Allosteric inhibition

Allosterically, regulation of these enzymes works by inducing different physical states (shapes, as it were) that affect their ability to bind to substrate. When an enzyme is inhibited by binding an effector, it is converted to the T-state (T=tight), it has a reduced affinity for substrate and it is through this means that the reaction is slowed.

Allosteric activation

On the other hand, when an enzyme is activated by effector binding, it converts to the R-state (R=relaxed) and binds substrate much more readily. When no effector is present, the enzyme may be in a mixture of T- and R-states.

Feedback inhibition

An interesting kind of allosteric control is exhibited by HMG-CoA reductase, which catalyzes an important reaction in the pathway leading to the synthesis of cholesterol. Binding of cholesterol to the enzyme reduces the enzyme's activity significantly. Cholesterol is not a substrate for the enzyme, so it is therefore a heterotropic effector.

Notably, though, cholesterol is the end-product of the pathway that HMG-CoA reductase catalyzes a reaction in. When enzymes are inhibited by an end-product of the pathway in which they participate, they are said to exhibit feedback inhibition.

Feedback inhibition always operates by allosterism and further, provides important and efficient control of an entire pathway. By inhibiting an early enzyme in a pathway, the flow of materials (and ATP hydrolysis required for their processing) for the entire pathway is stopped or reduced, assuming there are not alternate supply methods.

Pathway control

In the cholesterol biosynthesis pathway, stopping this one enzyme has the effect of shutting off (or at least slowing down) the entire pathway. This is significant because after catalysis by HMG-CoA reductase, there are over 20 further reactions necessary to make cholesterol, many of them requiring ATP energy. Shutting down one reactions stops all of them. Another excellent example of allosteric control and feedback inhibition is the enzyme ATCase, discussed below.

ATCase

Another interesting example of allosteric control and feedback inhibition is associated with the enzyme Aspartate Transcarbamoylase (ATCase). This enzyme, which catalyzes a step in the synthesis of pyrimidine nucleotides, has 12 subunits. These include six identical catalytic subunits and six identical regulatory subunits. The catalytic subunits bind to substrate and

catalyze a reaction. The regulatory subunits bind to either ATP or CTP. If they bind to ATP, the enzyme subunits arrange themselves in the R-state.

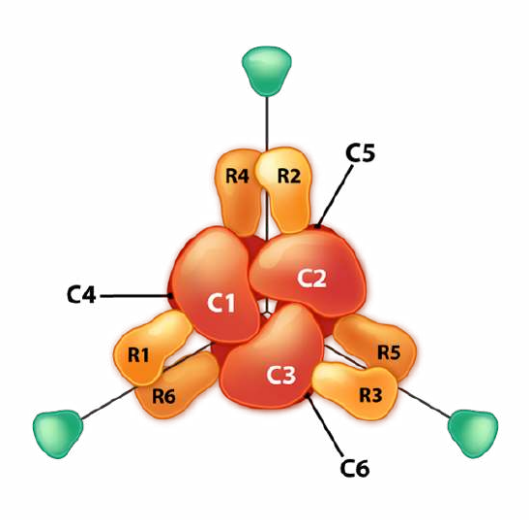


Figure 4.45 - Schematic structure of ATCase. Regulatory Units = R, Catalytic Units = C. Image by Aleia Kim

R-state

The R-state of ATCase allows the substrate to have easier access to the six active sites and the reaction occurs more rapidly. For the same amount of substrate, an enzyme in the R-state will have a higher velocity than the same enzyme that is not in the R-state. By contrast, if the enzyme binds to CTP on one of its regulatory subunits, the subunits will arrange in the T-state and in this form, the substrate will not have easy access to the active sites, resulting in a slower velocity for the same concentration of substrate compared to the R-state. ATCase is interesting in that it can also flip into the R-state when one of the substrates (aspartate) binds to an active site within one of the catalytic subunits.

Aspartate has the effect of activating the catalytic action of the enzyme by favoring the R-state. Thus, aspartate, which is a substrate of the enzyme is a homotropic effector and ATP and CTP, which are not substrates of the enzyme are heterotropic effectors of ATCase.

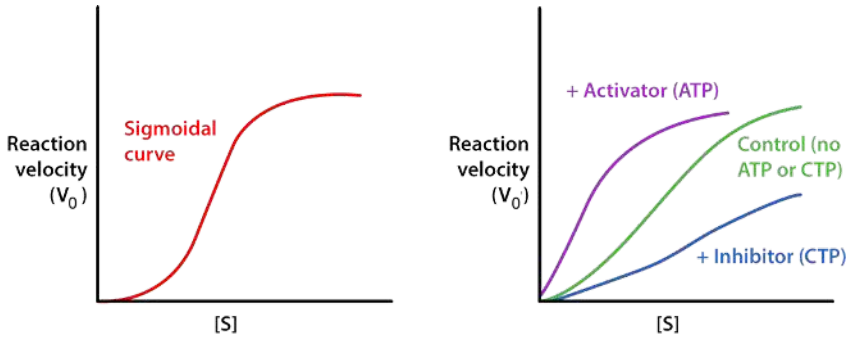


Figure 4.46 - Plots of V0 vs. [S] for ATCase. Left - Allosteric effect of aspartate. Right - Allosteric effects of ATP (activator) and CTP (inhibitor). Image by Pehr Jacobson

Optional: Allosteric models

There are three models commonly used to explain how allosterism regulates multi-subunit enzyme activity. They are known as

- the Monod-Wyman-Changeux (MWC) model (also known as the concerted model),
- the sequential model (also known as KNF),
- and the morpheein model.

All models describe a Tense (T) state that is less catalytically active and a Relaxed (R) state that is more catalytically active. The models differ in how the states change.

Sequential model

In the sequential model, binding of an allosteric effector by one subunit causes it to change from T to R state (or vice versa) and that change makes it easier for adjacent subunits to similarly change state. With this model , there is a cause/effect relationship between binding of an effector by one subunit and change of state by an adjacent subunit.

In hemoglobin, for example, binding of one oxygen by one unit of the complex may induce that unit to flip to the R-state and, through interactions with other subunits, cause them to favor adopting the R configuration before they bind to oxygen. In this way, binding of one subunit favors binding of others and cooperativity can be explained by the change in binding affinity as oxygen concentration changes.

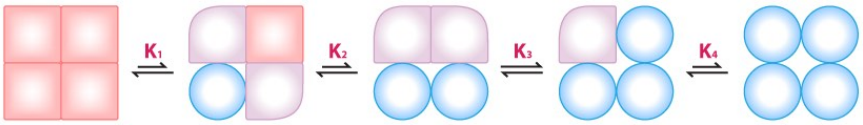


Figure 4.47 - Sequential model of allosteric regulation. Round = R-State. Square = T-state.. Image by Aleia Kim

MWC model

The MWC model is less intuitive. In it, the entire complex changes state from T to R (or vice versa) independently of the binding of effectors. Flipping between T-states and R-states is postulated to be in an equilibrium of states in the absence of effector (for example, a 50 to 1 ratio of T/R. This ratio is referred to as L, so L = T/R). Binding of effector by the enzyme complex has the tendency of “locking” the complex in a state. Binding of inhibitors will increase the ratio of T/R whereas binding of activators will increase R and thus decrease the ration of T/R.

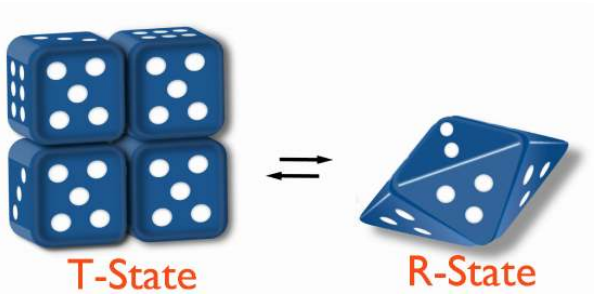


Figure 4.48 - The MWC Model - The multisubunit complex flips as a whole and is in equilibrium. Wikipedia

Morpheein model

The morpheein model is similar to the MWC model, but with an added step of dissociation of the subunits. The MWC model proposes that flipping between R and T states occurs by the complex as a whole and occurs on all units simultaneously. The morpheein model instead proposes that the multi-subunit enzyme breaks down to individual units which can then flip in structure and re-form the complex. In the morpheein model, only identically shaped units (all R, for example) can come together in the complex, thus explaining the “all-R-” or “all-T-” state found in the MWC model.

A large number of enzymes, including prominent ones like citrate synthase, acetyl-CoA carboxylase, glutamate dehydrogenase, ribonucleotide reductase and lactate dehydrogenase have behavior consistent with the morpheein model.



Figure 4.49 - Morpheein model of allosterism. States flip as monomers and aggregate only with same monomer. Wikipedia

Covalent control of enzymes

Some enzymes are synthesized in a completely inactive form and their activation requires covalent bonds in them to be cleaved. Such inactive forms of enzymes are called zymogens. Examples include the proteins involved in blood clotting and proteolytic enzymes of the digestive system, such as trypsin, chymotrypsin, pepsin, and others.

Synthesizing some enzymes in an inactive form makes very good sense when an enzyme's activity might be harmful to the tissue where it is being made. For example, the painful condition known as pancreatitis arises when digestive enzymes made in the pancreas are activated too soon and end up attacking the pancreas.

Cascades

For both the blood clotting enzymes and the digestive enzymes, the zymogens are activated in a protease cascade. This occurs when activation of one enzyme activates others in a sort of chain reaction. In such a scheme the first enzyme activated proteolytically cleaves the second zymogen, causing it to be activated, which in turn activates a third and this may proceed through several levels of enzymatic action (Figure 4.50).

The advantage of cascades is that they allow a large amount of zymogens to become activated fairly quickly, since there is an amplification of the signal at each level of catalysis.

Zymogens are also abundant in blood. Blood clotting involves polymerization of a protein known as fibrin. Since random formation of fibrin is extremely hazardous because it can block the flow of blood, potentially causing heart attack/stroke, the body synthesizes fibrin as a zymogen (fibrinogen) and its activation results from a "cascade" of activations of proteases that arise when a signal is received from a wound. Similarly, the enzyme catalyzing removal of fibrin clots (plasmin) is also synthesized as a zymogen (plasminogen), since random clot removal would also be hazardous (see below also).

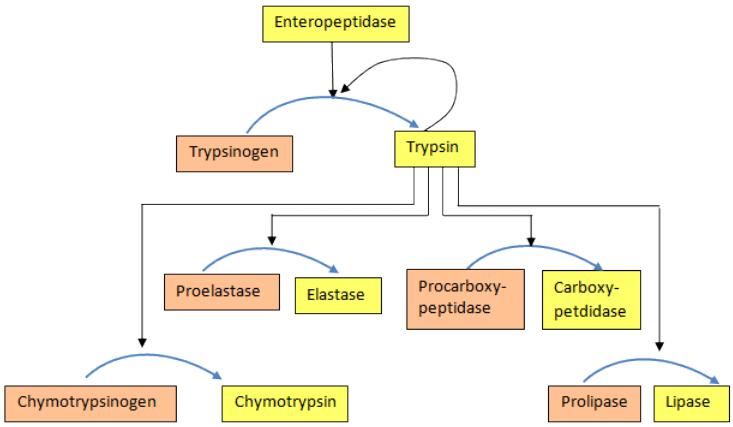


Figure 4.50 - Protease activation scheme. Wikipedia

Phosphorylation/dephosphorylation

Another common mechanism for control of enzyme activity by covalent modification is phosphorylation. The phosphorylation of enzymes (on the side chains of serine, threonine or tyrosine residues) is carried out by protein kinases. Enzymes activated by phosphorylation can be regulated by the addition of phosphate groups by kinases or their removal by phosphatases. Thus, this type of covalent modification is readily reversible, in contrast to proteolytic cleavage.

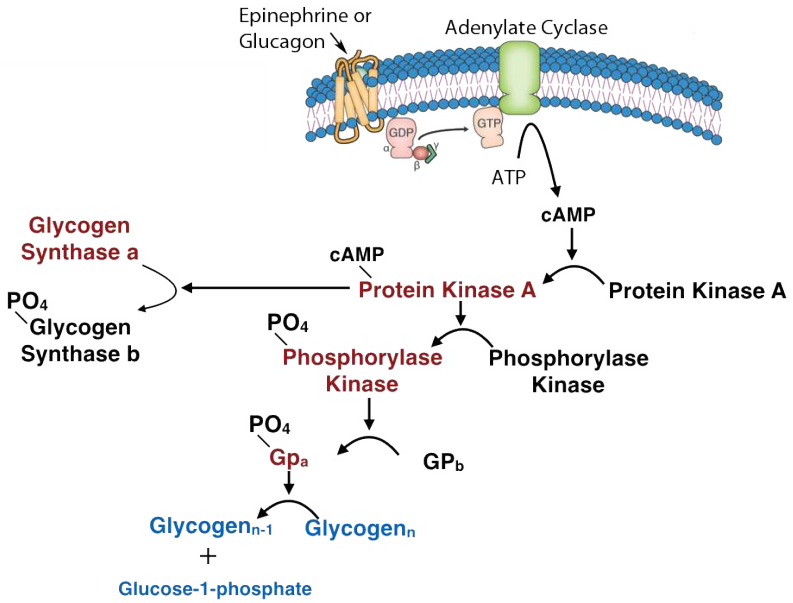


Figure 4.51 - Regulation by covalent modification of glycogen catabolism enzymes

Reduction/oxidation

An interesting covalent control of enzymes using reduction/oxidation is exhibited in photosynthetic plants. In the light phase of photosynthesis, electrons are excited by light and flow through carriers to NADP+, forming NADPH. Thus, in the light, the NADPH concentration is high. When NADPH concentration is high, the concentration of reduced ferredoxin (a molecule donating electrons to NADP+) is also high.

Reduced ferredoxin can transfer electrons to thioredoxin, reducing it. Reduced thioredoxin can, in turn, transfer electrons to proteins to reduce their disulfide bonds. Four enzymes related to the Calvin cycle can receive electrons from thioredoxin and become activated, as a result.

These include sedoheptulose 1,7-bisphosphatase, ribulose-5-phosphate kinase, fructose 1,6-bisphosphatase, and glyceraldehyde 3-phosphate dehydrogenase. Thus, in the light, electrons flow, causing NADPH to accumulate and ferredoxin to push electrons in the direction of these enzymes above, activating them and favoring the Calvin cycle. In the dark, the concentration of reduced NADPH, reduced ferredoxin, and reduced thioredoxin fall, resulting in loss of electrons by the Calvin cycle enzymes (oxidations that re-form disulfide bonds) and the Calvin cycle inactivates.

Other enzyme control mechanisms

Other means of controlling enzymes relate to access to the substrate (substrate-level control) and control of enzyme synthesis. Hexokinase is an enzyme that is largely regulated by the availability of its substrate, glucose. When glucose concentration is low, the product of the enzyme's catalysis, glucose-6-phosphate, inhibits the enzyme's function.

Regulation of enzymes by controlling their synthesis is covered later in the book in the discussion relating to control of gene expression.

This page titled 3.2: Control of Enzymatic Activity is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **4.2: Control of Enzymatic Activity** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

3.3: Mechanisms of Catalysis

A printable version of this section is here: BiochemFFA_4_3.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

The magic of enzymes, as noted, is in their ability to create electronic environments conducive to initiation of a reaction. There are more mechanisms of reaction than we could ever hope to cover in a book like this, and comprehensive discussion of these is not our aim. Instead, we will cite some examples and go into detail on one of them - the mechanism of action of serine proteases.

Chymotrypsin

We will begin with mechanism of action of one enzyme - chymotrypsin. Found in our digestive system, chymotrypsin's catalytic activity is cleaving peptide bonds in proteins and it uses the side chain of a serine in its mechanism of catalysis. Many other protein-cutting enzymes employ a very similar mechanism and they are known collectively as serine proteases (Figure 4.52).

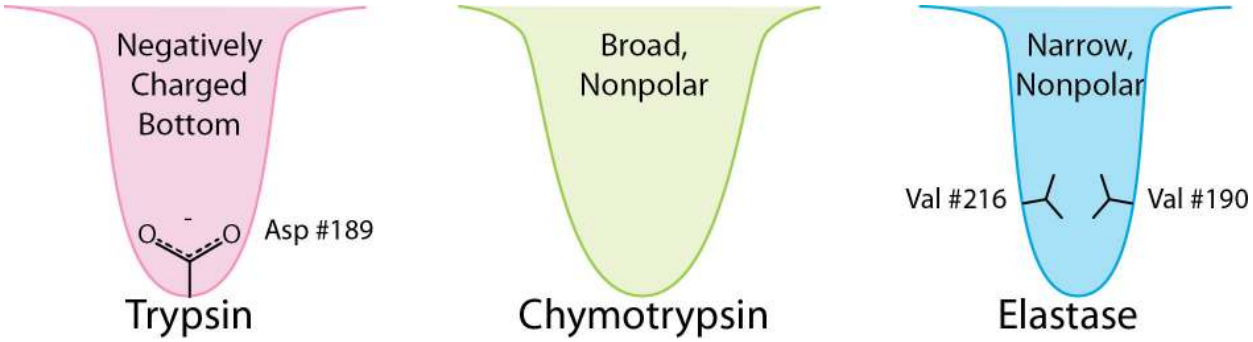


Figure 4.52 - Substrate binding sites (S1 pockets) of three serine proteases. Image by Aleia Kim

These enzymes are found in prokaryotic and eukaryotic cells and all use a common set of three amino acids in the active site called a catalytic triad (Figure 4.53). It consists of aspartic acid, histidine, and serine. The serine is activated in the reaction mechanism to form a nucleophile in these enzymes and gives the class their name. With the exception of the recognition that occurs at the substrate binding site, the mechanism shown here for chymotrypsin would be applicable to any of the serine proteases.

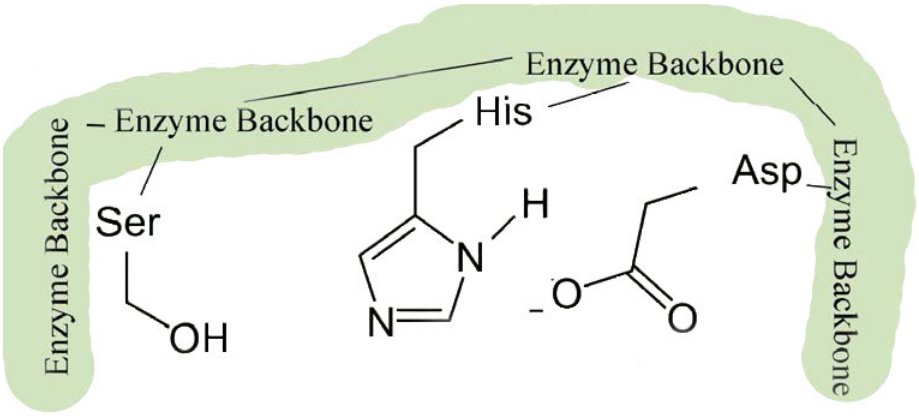


Figure 4.53 - 1. Active site of chymotrypsin showing the catalytic triad of serine - histidine-aspartic acid

Specificity

As a protease, chymotrypsin acts fairly specifically, cutting not all peptide bonds, but only those that are adjacent to relatively non-polar amino acids in the protein. One of the amino acids it cuts adjacent to is phenylalanine. The enzyme's action occurs in two phases – a fast phase that occurs first and a slower phase that follows. The enzyme has a substrate binding site that includes a region of the enzyme known as the S1 pocket. Let us step through the mechanism by which chymotrypsin cuts adjacent to phenylalanine.

Substrate binding

The process starts with the binding of the substrate in the S1 pocket (Figure 4.54). The S1 pocket in chymotrypsin has a hydrophobic hole in which the substrate is bound. Preferred substrates will include amino acid side chains that are bulky and hydrophobic, like phenylalanine. If an ionized side chain, like that of glutamic acid binds in the S1 pocket, it will quickly exit, much like water would avoid an oily interior.

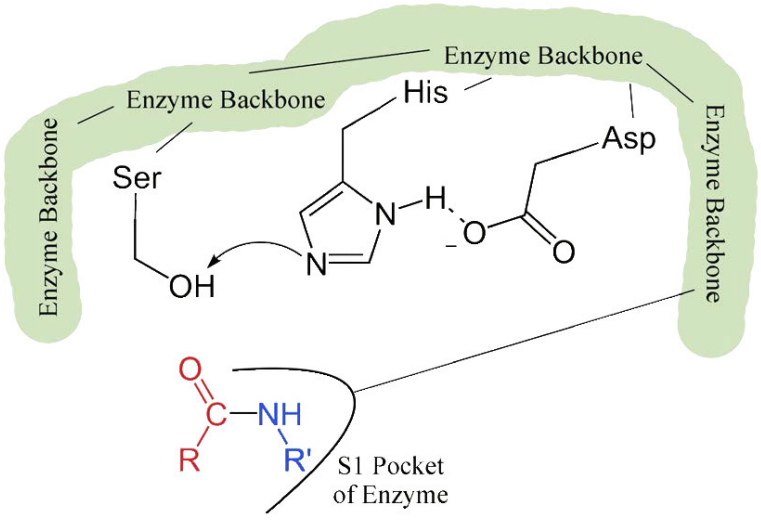


Figure 4.54 - 2. Binding of substrate to S1 pocket in the active site

Shape change on binding

When the proper substrate binds in the S1 pocket, its presence induces an ever so slight change in the shape of the enzyme. This subtle shape change on the binding of the proper substrate starts the steps of the catalysis. Since the catalytic process only starts when the proper substrate binds, this is the reason that the enzyme shows specificity for cutting at specific amino acids in the target protein. Only amino acids with the side chains that interact well with the S1 pocket start the catalytic wheels turning.

The slight changes in shape involve changes in the positioning of three amino acids (aspartic acid, histidine, and serine) in the active site known as the *catalytic triad*.

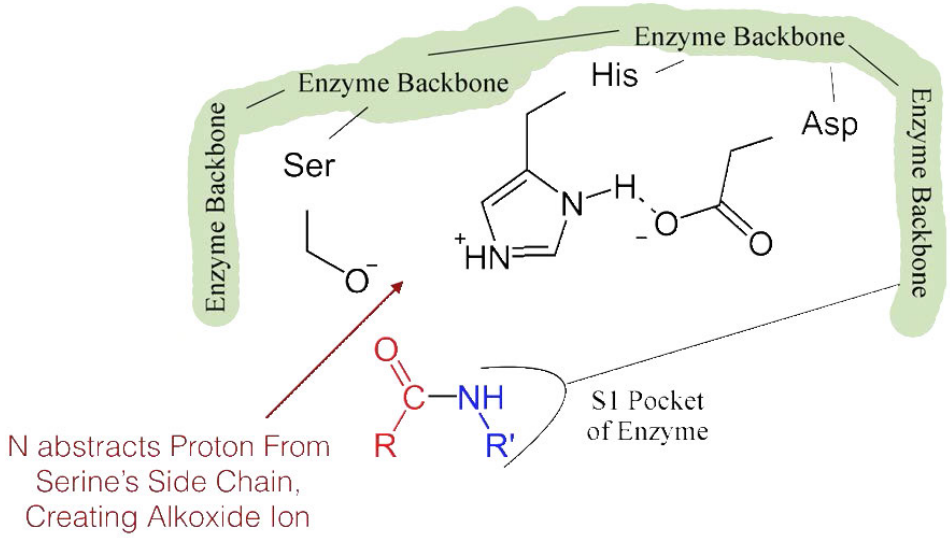


Figure 4.55 - 3. Formation of alkoxide ion

The shift of the negatively charged aspartic acid towards the electron rich histidine ring favors the abstraction of a proton by the histidine from the hydroxyl group on the side chain of serine, resulting in production of a very reactive alkoxide ion in the active

site (Figure 4.55).

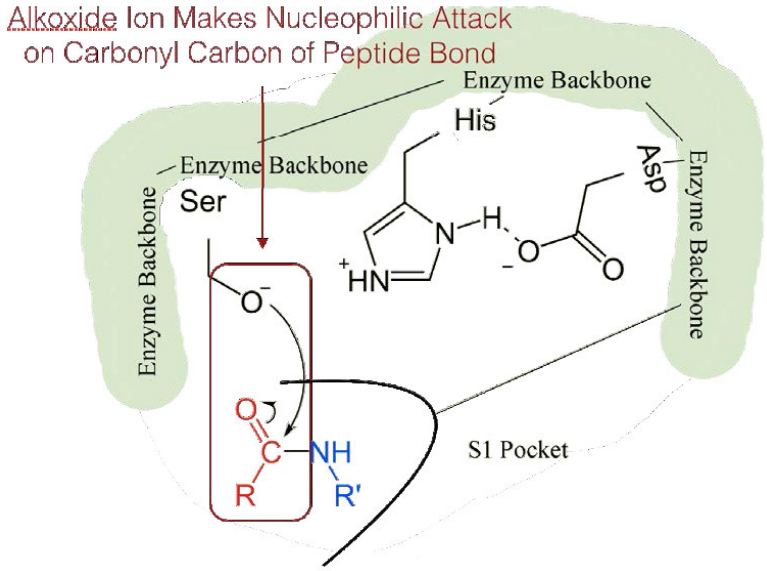


Figure 4.56 - 4. Nucleophilic attack

Since the active site at this point also contains the polypeptide chain positioned with the phenylalanine side chain embedded in the S1 pocket, the alkoxide ion performs a nucleophilic attack on the peptide bond on the carboxyl side of phenylalanine sitting in the S1 pocket (Figure 4.56). This reaction breaks the peptide bond (Figure 4.57) and causes two things to happen.

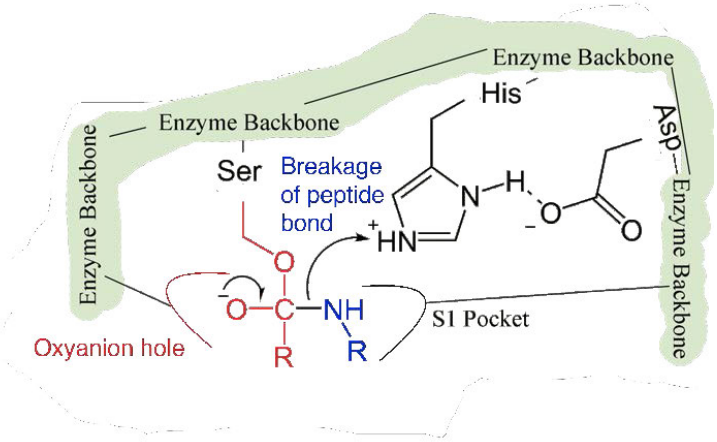


Figure 4.57 - 5. Stabilization by oxyanion hole. Breakage of peptide bond.

First, one end of the original polypeptide is freed and exits the active site (Figure 4.58). The second is that the end containing the phenylalanine is covalently linked to the oxygen of the serine side chain. At this point we have completed the first (fast) phase of the catalysis.

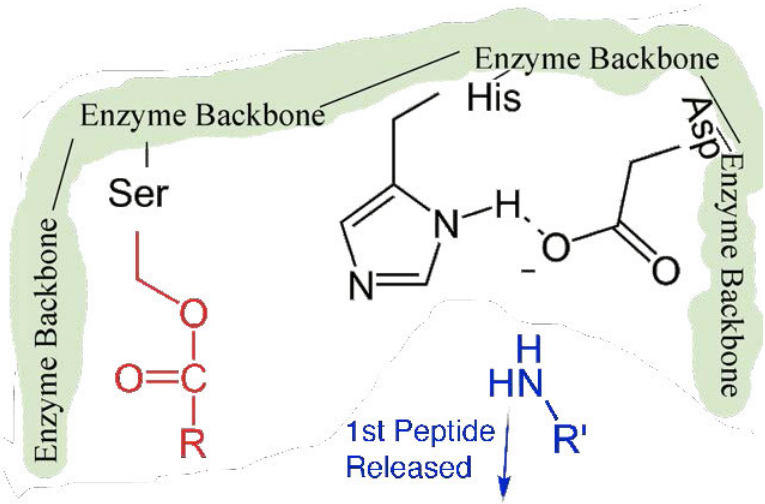


Figure 4.58 - 6. First peptide released. Other half bonded to serine.

Slower second phase

The second phase of the catalysis by chymotrypsin is slower. It requires that the covalent bond between phenylalanine and serine's oxygen be broken so the peptide can be released and the enzyme can return to its original state. The process starts with entry of water into the active site. Water is attacked in a fashion similar to that of the serine side chain in the first phase, creating a reactive hydroxyl group (Figure 4.59) that performs a nucleophilic attack on the phenylalanine-serine bond (Figure 4.60), releasing it and replacing the proton on serine. The second peptide is released in the process and the reaction is complete with the enzyme back in its original state (Figure 4.61).

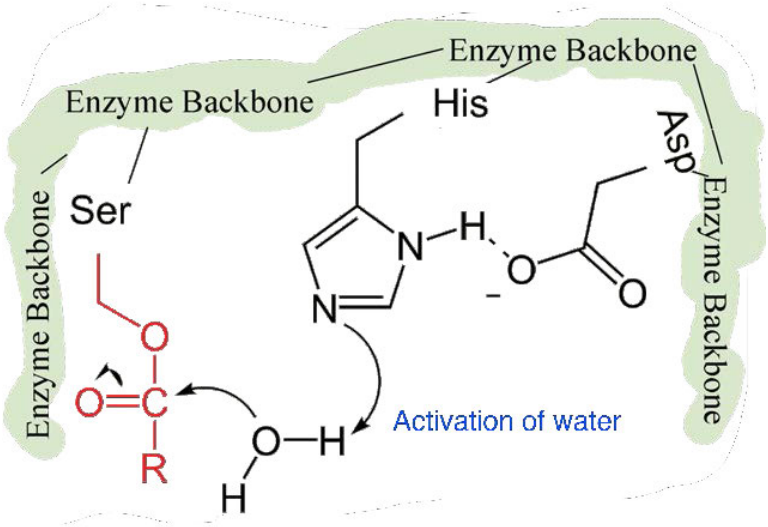


Figure 4.59 - 7. Activation of water by histidine

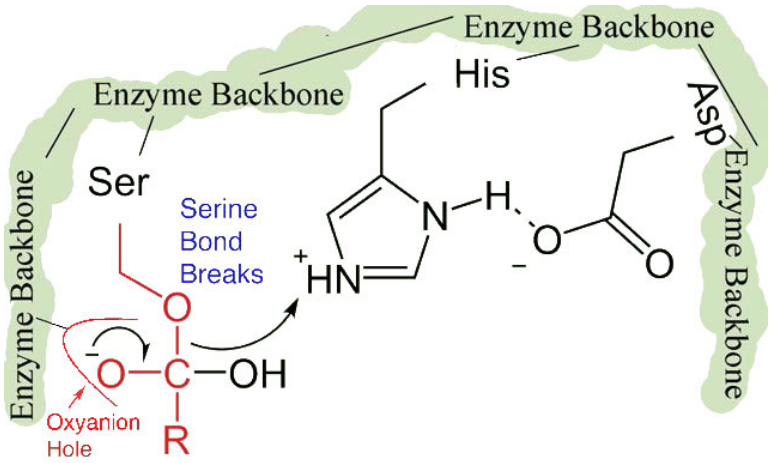


Figure 4.60 - 8. Nucleophile attack by hydroxyl creates tetrahydryl intermediate stabilized by oxyanion hole. Bond to serine breaks.

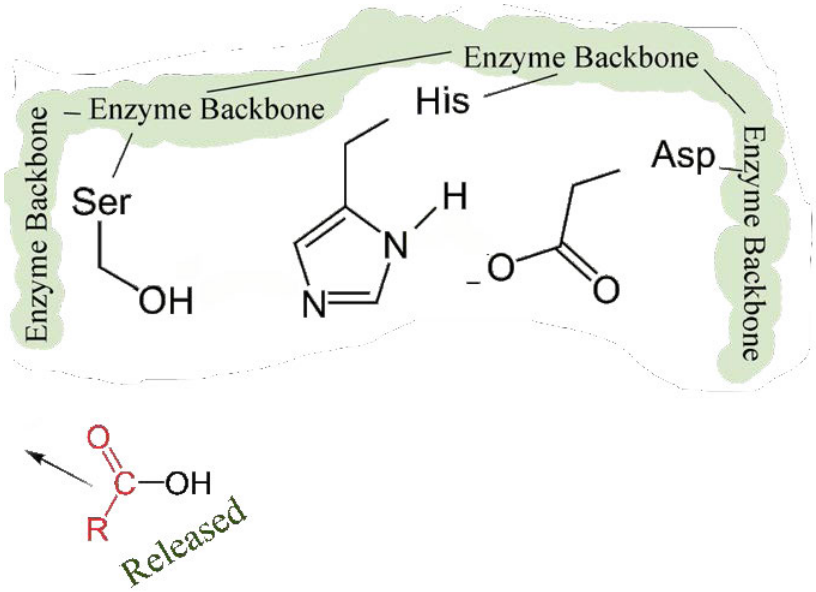


Figure 4.61 - 9. Second half of peptide released. Enzyme active site restored.

Serine proteases

The list of serine proteases is quite long. They are grouped in two broad categories - 1) those that are chymotrypsin-like and 2) those that are subtilisin-like. Though subtilisin-type and chymotrypsin-like enzymes use the same mechanism of action, including the catalytic triad, the enzymes are otherwise not related to each other by sequence and appear to have evolved independently. They are, thus, an example of convergent evolution - a process where evolution of different forms converge on a structure to provide a common function.

The serine protease enzymes cut adjacent to specific amino acids and the specificity is determined by the size/shape/charge of amino acid side chain that fits into the enzyme's S1 binding pocket (Figure 4.62).

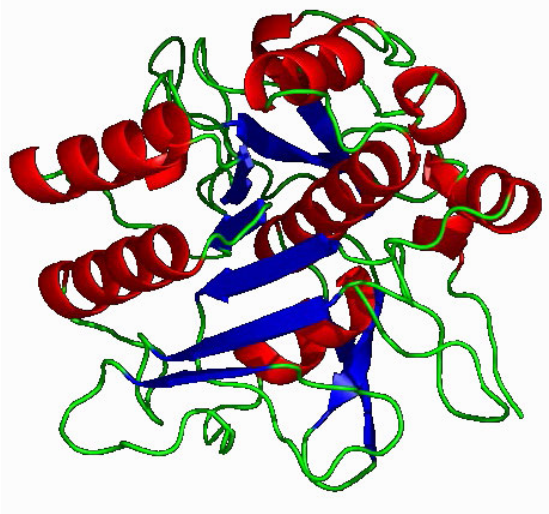


Figure 4.62 - Subtilisin - A serine protease

Examples of serine proteases include trypsin, chymotrypsin, elastase, subtilisin, signal peptidase I, and nucleoporin. Serine proteases participate in many physiological processes, including blood coagulation, digestion, reproduction, and the immune response.

Cysteine proteases

Cysteine proteases (also known as thiol proteases) catalyze the breakdown of proteins by cleaving peptide bonds using a nucleophilic thiol from a cysteine (Figure 4.63). The cysteine is typically found in a catalytic dyad or triad also involving histidine and (sometimes) aspartic acid (very much like serine proteases). The sulfhydryl group of cysteine proteases is more acidic than the hydroxyl of serine proteases, so the aspartic acid of the triad is not always needed.

The mechanism of action is very similar to that of serine proteases. Binding of proper substrate results in activation of the thiol (removal of the proton by the histidine group). The activated thiol acts as a nucleophile, attacking the peptide bond and causing it break. One peptide is released and the other peptide becomes covalently linked to the sulfur. Hydrolysis by water releases the second peptide and completes the cycle. Examples of cysteine proteases include papain, caspases, hedgehog protein, calpain, and cathepsin K.

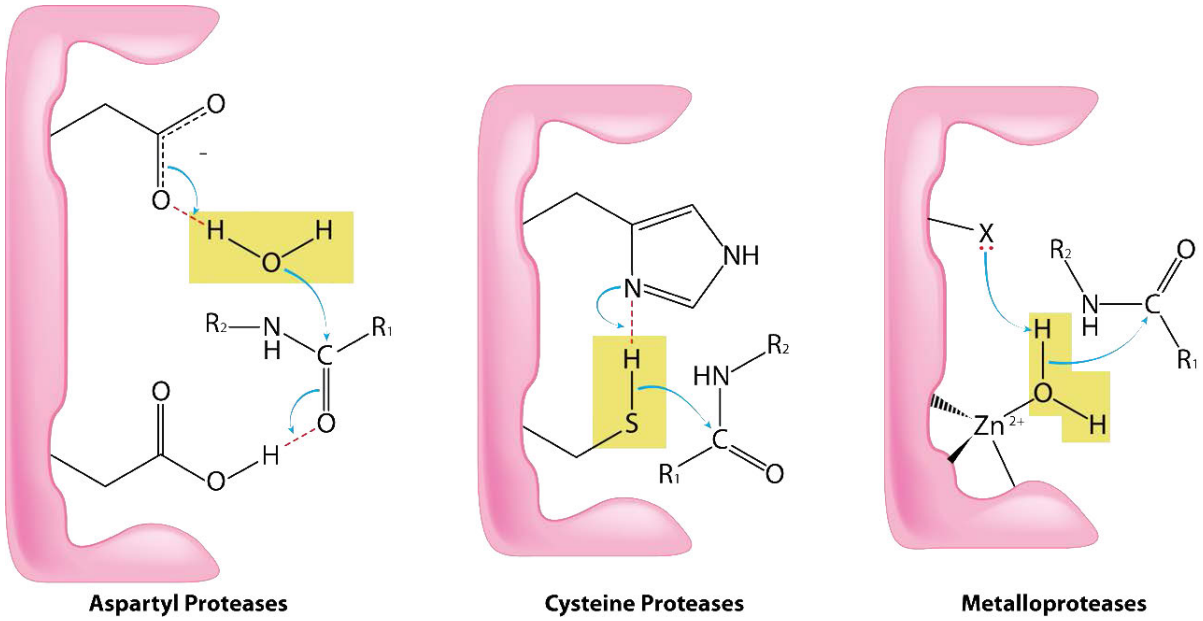


Figure 4.63 - Mechanism of action of proteases. In each case, a nucleophile is created - hydroxyl (aspartyl proteases), thiol (cysteine proteases), and hydroxyl (metalloproteases). Image by Aleia Kim

Caspases

Caspases (Cysteine-ASPartic ProteASEs) are a family of cysteine proteases that play important roles in the body. At the cellular level they function in apoptosis and necrosis and in the body, they are involved in inflammation and the immune system. Maturation of lymphocytes is one such role. They are best known, however, for their role in apoptosis, which has given rise to descriptions of them as “executioner” proteins or “suicide proteases” that dismantle cells in programmed cell death.

There are 12 known human caspases. The enzymes are synthesized as pro-caspase zymogens with a prodomain and two other subunits. The prodomain contains regions that allow it to interact with other molecules that regulate the enzyme’s activity. The caspases come in two forms. The initiator caspases, when activated, activate the effector caspases. The effector caspases cleave other proteins in the cell. Targets for effector caspase cleavage action include the nuclear lamins (fibrous proteins providing structural integrity to the nucleus), ICAD/DFF45 (an inhibitor of DNAse), PARP (flags areas where DNA repair needed), and PAK2 (apoptotic regulation).

The caspase activation cascade can itself be activated by granzyme B (a serine protease secreted by natural killer cells and cytotoxic T-cells), cellular death receptors, and the apoptosome (large protein structure in apoptotic cells stimulated by release of cytochrome C from the mitochondria). Each of these activators is responsible for activating different groups of caspases.

Metalloproteases

Metalloproteases (Figure 4.64) are enzymes whose catalytic mechanism for breaking peptide bonds involves a metal. Most metalloproteases use zinc as their metal, but a few use cobalt, coordinated to the protein by three amino acid residues with a labile water at the fourth position. A variety of side chains are used - histidine, aspartate, glutamate, arginine, and lysine. The water is the target of action of the metal which, upon binding of the proper substrate, abstracts a proton to create a nucleophilic hydroxyl group that attacks the peptide bond, cleaving it (Figure 4.64). Since the nucleophile here is not attached covalently to the enzyme, neither of the cleaved peptides ends up attached to the enzyme during the catalytic process. Examples of metalloproteases include carboxypeptidases, aminopeptidases, insulinases and thermolysin.



Figure 4.64 - Carboxypeptidase - A metalloprotease

Aspartyl proteases

As the name suggests, aspartyl proteases use aspartic acid in their catalytic mechanism (Figures 4.63 & 4.65). Like the metalloproteases, aspartyl proteases activate a water to create a nucleophile for catalysis (Figure 4.65). The activated water attacks the peptide bond of the bound substrate and releases the two pieces without the need to release a bound intermediate, since water is not covalently attached to the enzyme. Common aspartyl proteases include pepsin, signal peptidase II, and HIV-1 protease.

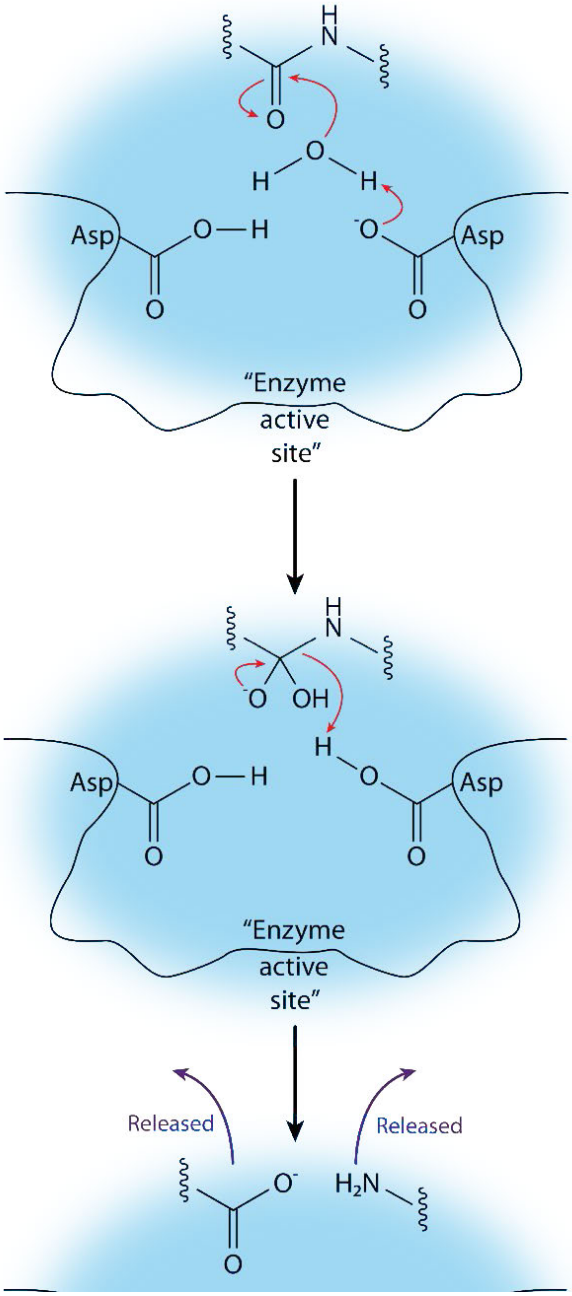


Figure 4.65 - Activation of an aspartyl protease - aspartate side chain abstracts proton (top). Hydroxyl attacks peptide bond (middle). Broken peptide pieces released (bottom). Image by Pehr Jacobson

Threonine proteases

Though threonine has an R-group with a hydroxyl like serine, the mechanism of action of this class of proteases differs somewhat from the serine proteases. There are some similarities. First, the threonine's hydroxyl plays a role in catalysis and that is to act as a nucleophile. The nucleophile is created, however, not by a catalytic triad, but rather as a result of threonine's own α-amine group abstracting a proton.

Because of this, the nucleophilic threonine in a threonine protease must be at the n-terminus of the enzyme. Nucleophilic attack of the peptide bond in the target protease results in breakage of the bond to release one peptide and the other is covalently attached to serine, like the serine proteases. Also, as with the serine proteases, water must come in to release the covalently linked second peptide to conclude the catalytic mechanism.

Examples

Examples of threonine proteases include the catalytic subunits of the proteasome. Some acyl transferases (such as ornithine acyltransferase) have evolved the same catalytic mechanism by convergent evolution. The latter enzymes use ornithine instead of water to break the enzyme-substrate covalent bond, with the result that the acyl-group becomes attached to ornithine, instead of water.

Protease inhibitors

Molecules which inhibit the catalytic action of proteases are known as protease inhibitors. These come in a variety of forms and have biological and medicinal uses. Many biological inhibitors are proteins themselves. Protease inhibitors can act in several ways, including as a suicide inhibitor, a transition state inhibitor, a denaturant, and as a chelating agent. Some work only on specific classes of enzymes. For example, most known aspartyl proteases are inhibited by pepstatin. Metalloproteases are sensitive to anything that removes the metal they require for catalysis. Zinc-containing metalloproteases, for example, are very sensitive to EDTA, which chelates the zinc ion.

One category of proteinaceous protease inhibitors is known as the serpins. Serpins inhibit serine proteases that act like chymotrypsin. 36 of them are known in humans.

Serpins are unusual in acting by binding to a target protease irreversibly and undergoing a conformational change to alter the active site of its target. Other protease inhibitors act as competitive inhibitors that block the active site.

Serpins can be broad in their specificity. Some, for example, can block the activity of cysteine proteases. One of the best known biological serpins is α-1-anti-trypsin (A1AT - Figure 4.66) because of its role in lungs, where it functions to inhibit the elastase protease. Deficiency of A1AT leads to emphysema. This can arise as a result of genetic deficiency or by cigarette smoking. Reactive oxygen species produced by cigarette smoking can oxidize a critical methionine residue (#358 of the processed form) in A1AT, rendering it unable to inhibit elastase. Uninhibited, elastase can attack lung tissue and cause emphysema. Most serpins work extracellularly. In blood, for example, serpins like antithrombin can help to regulate the clotting process.

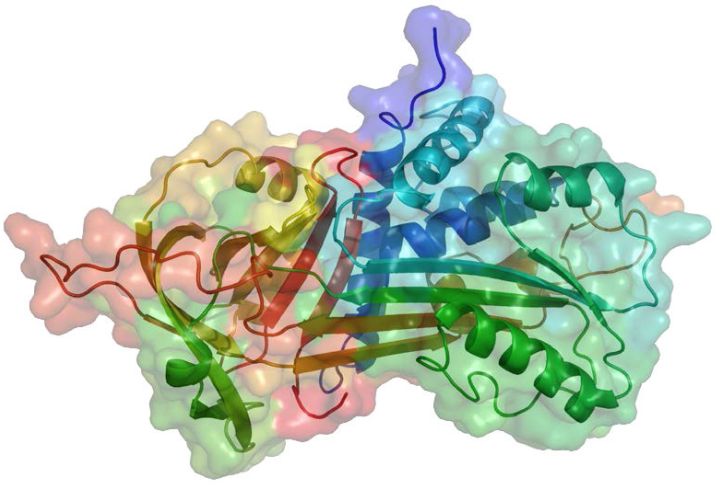


Figure 4.66 - α-1-antitrypsin

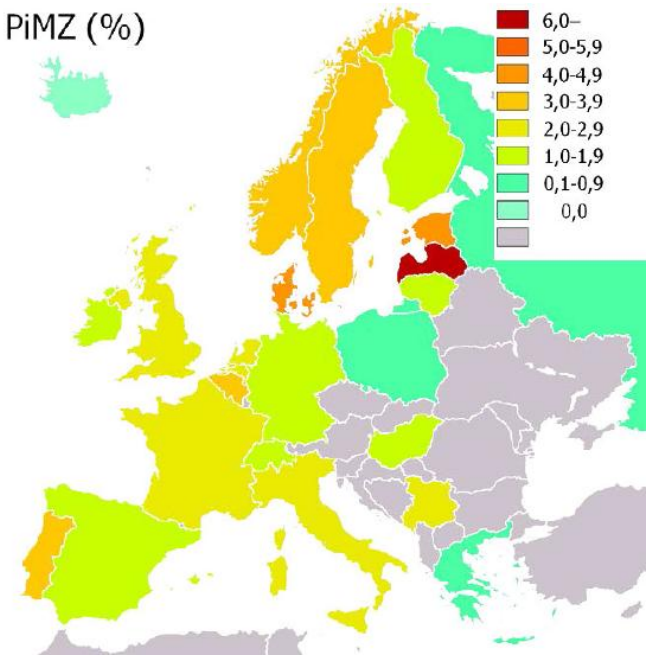


Figure 4.67 - Incidence of α-1-antitrypsin (PiMZ) deficiency in Europe by percent. Wikipedia

Anti-viral Agents

Protease inhibitors are used as anti-viral agents to prohibit maturation of viral proteins - commonly viral coat proteins.

They are part of drug “cocktails” used to inhibit the spread of HIV in the body and are also used to treat other viral infections, including hepatitis C. They have also been investigated for use in treatment of malaria and may have some application in anti-cancer therapies as well.

This page titled 3.3: Mechanisms of Catalysis is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **4.3: Mechanisms of Catalysis** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

CHAPTER OVERVIEW

4: Structure and Function- Nucleic Acids

4.1: Prelude to Information Processing
4.2: Structure and Function - Nucleic Acids
4.3: DNA Replication
4.4: DNA Repair
4.5: Genes and Genomes
4.6: Transcription
4.6.1: RNA Processing
4.7: RNA Processing
4.8: Translation
4.9: Gene Expression

4: Structure and Function- Nucleic Acids is shared under a not declared license and was authored, remixed, and/or curated by LibreTexts.

4.1: Prelude to Information Processing

“The blueprints for the construction of one human being requires only a meter of DNA and one tiny cell. … even Mozart started out this way.” — L.L. Larison Cudmore

As creatures used to regarding ourselves as exceptional, humans must surely be humbled to realize that the instructions, for making one of our own, reside in a molecule so simple that scientists, for a very long time, did not believe could possibly contain enough information to build even a simple cell. But a large body of evidence, built up over the past century, supports Larison Cudmore’s assertion that the information for making you and me (and all the other kinds of living things in the world) is encoded in DNA. Tying in with Mendel’s observations about how characteristics are passed on from one generation to the next, the discovery that there was a molecule that carried this information, altered for ever how people thought about heredity.

The elucidation of the structure of DNA provided greater insights into how traits might be encoded in a molecule, and the ways in which the information is used by cells. As we learn more about this topic, scientists have remarked on how the information in our DNA resembles the programs that drive computers. While this analogy is a simplification, there is definitely a sense in which, as Richard Dawkins put it, “the machine code of the genes is uncannily computer-like”, with information in our DNA directly determining the properties of the proteins that run our cells. We know, as Ada Yonath described it, that, “DNA is a code of four letters; proteins are made up of amino acids which come in 20 forms. So the ribosome is a very clever machine that reads one language and operates in another. “

If this sounds strange, it is even more intriguing to realize DNA is copied and passed on from cell to cell, from one generation to the next. There is an unbroken line of inheritance from the first cell to every organism alive today. In the words of Lewis Thomas, “All of today’s DNA, strung through all the cells of the earth, is simply an extension and elaboration of [the] first molecule.”

The nature of this information, how it is copied and passed on, how it is read and interpreted, and how it gives rise to the cellular activities that we can observe, is the subject of this chapter. Another kind of information is also considered, towards the end of the chapter- the molecular information that cells receive from, and send to, each other. Overlaid on the instructions in the genes, this information provides cells with ongoing clues about both their own inner state and the environment around them. The interplay of these two kinds of information is responsible for the form and behavior of all living organisms.

This page titled 4.1: Prelude to Information Processing is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **7.1: Prelude to Information Processing** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

4.2: Structure and Function - Nucleic Acids

Source: BiochemFFA_2_5.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

The nucleic acids, DNA and RNA, may be thought of as the information molecules of the cell. In this section, we will examine the structures of DNA and RNA, and how these structures are related to the functions these molecules perform.



We will begin with DNA, which is the hereditary information in every cell, that is copied and passed on from generation to generation. The race to elucidate the structure of DNA was one of the greatest stories of 20th century science. Discovered in 1869 by Friedrich Miescher, DNA was identified as the genetic material in experiments in the 1940s led by Oswald Avery, Colin MacLeod, and Maclyn McCarty. X-ray diffraction work of Rosalind Franklin and the observations of Erwin Chargaff were combined by James Watson and Francis Crick to form a model of DNA that we are familiar with today. Their famous paper, in the April 25, 1953 issue of Nature, opened the modern era of molecular biology. Arguably, that one-page paper has had more scientific impact per word than any other research article ever published. Today, every high school biology student is familiar with the double helical structure of DNA and knows that G pairs with C and A with T.

The double helix, made up of a pair of DNA strands, has at its core, bases joined by hydrogen bonds to form base pairs - adenine always paired with thymine, and guanine invariably paired with cytosine. Two hydrogen bonds are formed between adenine and thymine, but three hydrogen bonds hold together guanine and cytosine (Figure 2.127).

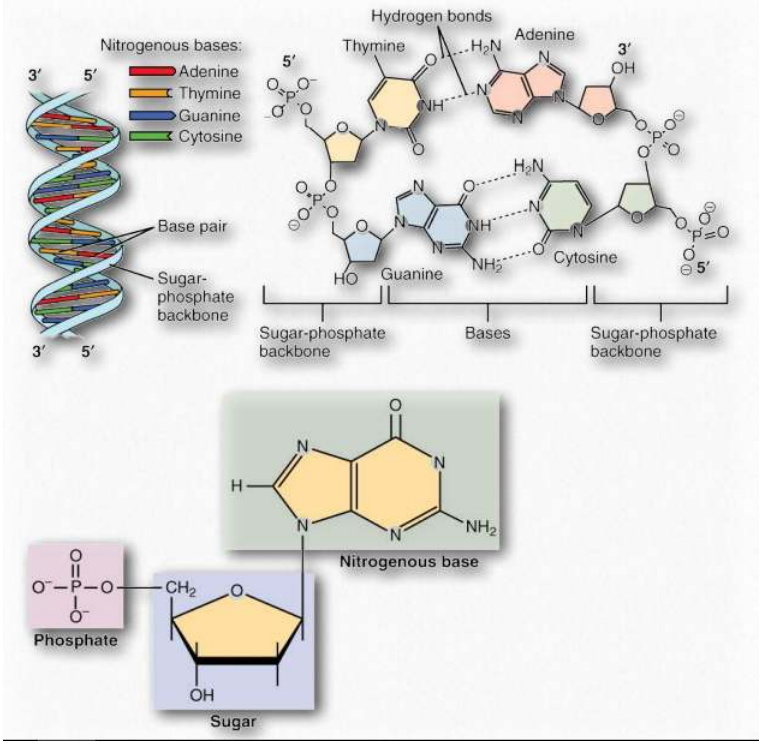


Figure 2.127 - A DNA duplex with base pairs, a closeup of base pairing, and a closeup of a nucleotide Wikipedia

The complementary structure immediately suggested to Watson and Crick how DNA might be (and in fact, is) replicated and it further explains how information is DNA is transmitted to RNA for the synthesis of proteins. In addition to the hydrogen bonds between bases of each strand, the double helix is held together by hydrophobic interactions of the stacked, non-polar bases. Crucially, the sequence of the bases in DNA carry the information for making proteins. Read in groups of three, the sequence of the bases directly specifies the sequence of the amino acids in the encoded protein.

Structure

A DNA strand is a polymer of nucleoside monophosphates held together by phosphodiester bonds. Two such paired strands make up the DNA molecule, which is then twisted into a helix. In the most common Bform, the DNA helix has a repeat of 10.5 base pairs per turn, with sugars and phosphate forming the covalent phosphodiester “backbone” of the molecule and the adenine, guanine, cytosine, and thymine bases oriented in the middle where they form the now familiar base pairs that look like the rungs of a ladder.

Building blocks

The term nucleotide refers to the building blocks of both DNA (deoxyribonucleoside triphosphates, dNTPs) and RNA (ribonucleoside triphosphates, NTPs). In order to discuss this important group of molecules, it is necessary to define some terms.

Nucleotides contain three primary structural components. These are a nitrogenous base, a pentose sugar, and at least one phosphate. Molecules that contain only a sugar and a nitrogenous base (no phosphate) are called nucleosides. The nitrogenous bases found in nucleic acids include adenine and guanine (called purines) and cytosine, uracil, or thymine (called pyrimidines). There are two sugars found in nucleotides - deoxyribose and ribose (Figure 2.128). By convention, the carbons on these sugars are labeled 1’ to 5’. (This is to distinguish the carbons on the sugars from those on the bases, which have their carbons simply labeled as 1, 2, 3, etc.) Deoxyribose differs from ribose at the 2’ position, with ribose having an OH group, where deoxyribose has H.

https://chem.libretexts.org/@go/page/347424

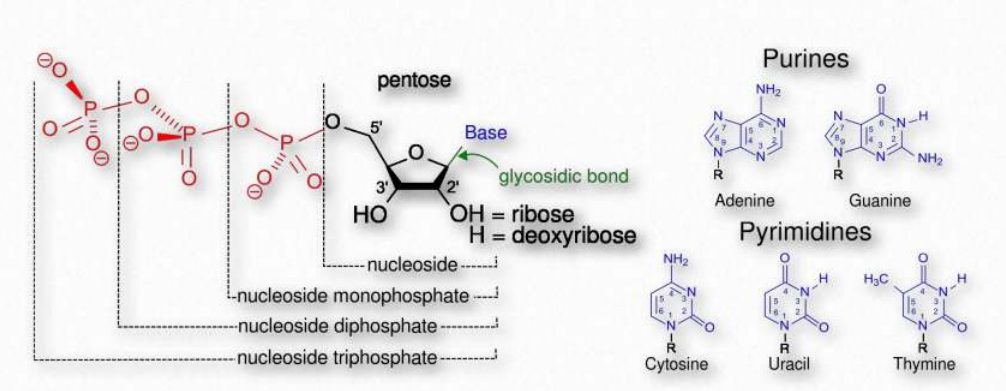


Figure 2.128 - Nucleotides, nucleosides, and bases

Nucleotides containing deoxyribose are called deoxyribonucleotides and are the forms found in DNA. Nucleotides containing ribose are called ribonucleotides and are found in RNA. Both DNA and RNA contain nucleotides with adenine, guanine, and cytosine, but with very minor exceptions, RNA contains uracil nucleotides, whereas DNA contains thymine nucleotides. When a base is attached to a sugar, the product, a nucleoside, gains a new name.

- **uracil-containing** = uridine (attached to ribose) / deoxyuridine (attached to deoxyribose)
- **thymine-containing** = ribothymidine (attached to ribose) / thymidine (attached to deoxyribose)
- **cytosine-containing** = cytidine (attached to ribose - Figure 2.129) / deoxycytidine (attached to deoxyribose)
- **guanine-containing** = guanosine (attached to ribose) / deoxyguanosine (attached to deoxyribose)
- **adenine-containing** = adenosine (attached to ribose) / deoxyadenosine (attached to deoxyribose)

Of these, deoxyuridine and ribothymidine are the least common. The addition of one or more phosphates to a nucleoside makes it a nucleotide. Nucleotides are often referred to as nucleoside phosphates, for this reason. The number of phosphates in the nucleotide is indicated by the appropriate prefixes (mono, di or tri).

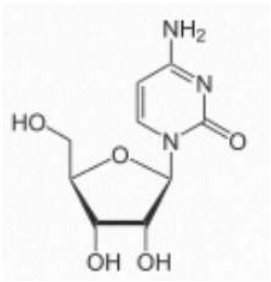


Figure 2.129 Cytidine

Thus, cytidine, for example, refers to a nucleoside (no phosphate), but cytidine monophosphate refers to a nucleotide (with one phosphate). Addition of second and third phosphates to a nucleoside monophosphate requires energy, due to the repulsion of negatively charged phosphates and this chemical energy is the basis of the high energy triphosphate nucleotides (such as ATP) that fuel cells.

Note: Ribonucleotides as Energy Sources

Though ATP is the most common and best known cellular energy source, each of the four ribonucleotides plays important roles in providing energy. GTP, for example, is the energy source for protein synthesis (translation) as well as for a handful of metabolic reactions. A bond between UDP and glucose makes UDP-glucose, the building block for making glycogen. CDP is similarly linked to several different molecular building blocks important for glycerophospholipid synthesis (such as CDP-diacylglycerol).

The bulk of ATP made in cells is not from directly coupled biochemical metabolism, but rather by the combined processes of electron transport and oxidative phosphorylation in mitochondria and/or photophosphorylation that occurs in the chloroplasts of photosynthetic organisms. Triphosphate energy in ATP is transferred to the other nucleosides/nucleotides by action of enzymes called kinases. For example, nucleoside diphosphokinase (NDPK) catalyzes the following reaction

$$ATP + NDP \longleftrightarrow ADP + NTP \quad (4.2.1)$$

where ‘N’ of “NDP” and “NTP corresponds to any base. Other kinases can put single phosphates onto nucleosides or onto nucleoside monophosphates using energy from ATP.

Deoxyribonucleotides

Individual deoxyribonucleotides are derived from corresponding ribonucleoside diphosphates via catalysis by the enzyme known as ribonucleotide reductase (RNR). The deoxyribonucleoside diphosphates are then converted to the corresponding triphosphates (dNTPs) by the addition of a phosphate group. Synthesis of nucleotides containing thymine is distinct from synthesis of all of the other nucleotides and will be discussed later.

Building DNA strands

Each DNA strand is built from dNTPs by the formation of a phosphodiester bond, catalyzed by DNA polymerase, between the 3’OH of one nucleotide and the 5’ phosphate of the next. The result of this directional growth of the strand is that the one end of the strand has a free 5’ phosphate and the other a free 3’ hydroxyl group (Figure 2.130). These are designated as the 5’ and 3’ ends of the strand.

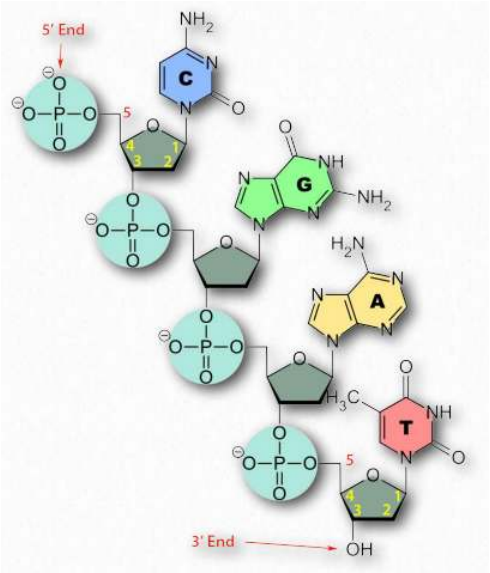


Figure 2.130 - 5’-3’ Polarity of a DNA strand

Figure 2.131 shows two strands of DNA (left and right). The strand on the left, from 5’ to 3’ reads T-C-G-A, whereas the strand on the right, reading from 5’ to 3’ is T-C-G-A. The strands in a double-stranded DNA are arranged in an anti-parallel fashion with the 5’ end of one strand across from the 3’ end of the other.

Hydrogen bonds

Hydrogen bonds between the base pairs hold a nucleic acid duplex together, with two hydrogen bonds per A-T pair (or per A-U pair in RNA) and three hydrogen bonds per G-C pair. The B-form of DNA has a prominent major groove and a minor groove tracing the path of the helix (Figure 2.132). Proteins, such as transcription factors bind in these grooves and access the hydrogen bonds of the base pairs to “read” the sequence therein.

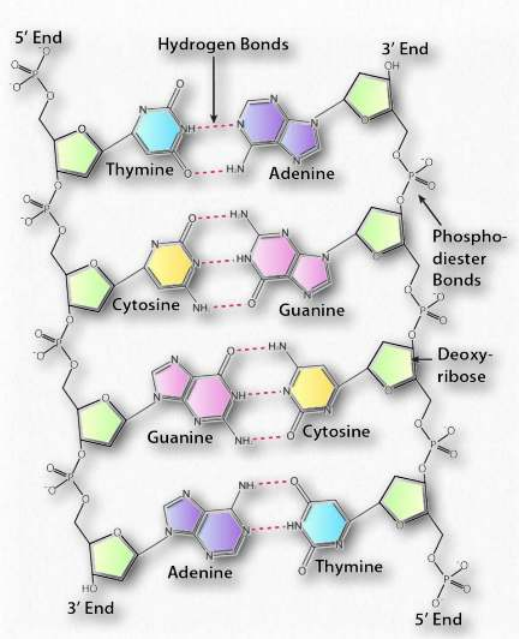


Figure 2.131 - Anti-parallel orientation of a DNA duplex, phosphodiester backbone, and base pairing Image by Aleia Kim

Other forms of DNA besides the B-form (Movie 2.5) are known (Figure 2.133). One of these, the A-form, was identified by Rosalind Franklin in the same issue of Nature as Watson and Crick's paper. Though the A-form structure is a relatively minor form of DNA and resembles the B-form, it turns out to be important in the duplex form of RNA and in RNA-DNA hybrids. Both the A form and the B-form of DNA have the helix oriented in what is termed the right-handed form.

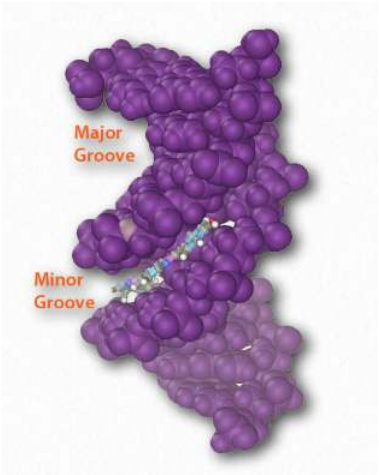
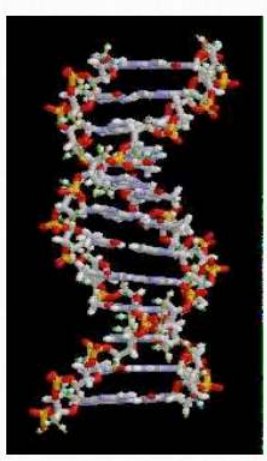


Figure 2.132 - Major and minor grooves of DNA. The minor groove has been bound by a dye Wikipedia



Movie 2.5 - B-form DNA duplex rotating in space Wikipedia

Z-DNA

The A-form and the B-form stand in contrast to another form of DNA, known as the Z-form. ZDNA, as it is known, has the same base-pairing rules as the B and A forms, but instead has the helices twisted in the opposite direction, making a left-handed helix (Figure 2.133). The Z-form has a sort of zig-zag shape, giving rise to the name Z-DNA.

In addition, the helix is rather stretched out compared to the A- and B-forms. Why are there different topological forms of DNA? The answer relates to both superhelical tension and sequence bias. Sequence bias means that certain sequences tend to favor the "flipping" of Bform DNA into other forms. ZDNA forms are favored by long stretches of alternating Gs and Cs. Superhelical tension is discussed below.

Superhelicity

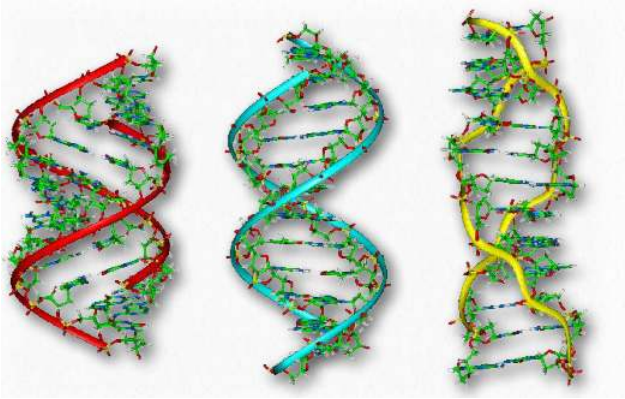


Figure 2.133 - From left to right, the A-, B-, and Zforms of DNA

Short stretches of linear DNA duplexes exist in the B-form and have 10.5 base pairs per turn. Double helices of DNA in the cell can vary in the number of base pairs per turn they contain. There are several reasons for this. For example, during DNA replication, strands of DNA at the site of replication get unwound at the rate of 6000 rpm by an enzyme called helicase. The effect of such local unwinding at one place in a DNA has the effect increasing the winding ahead of it. Unrelieved, such 'tension' in a DNA duplex can result in structural obstacles to replication.

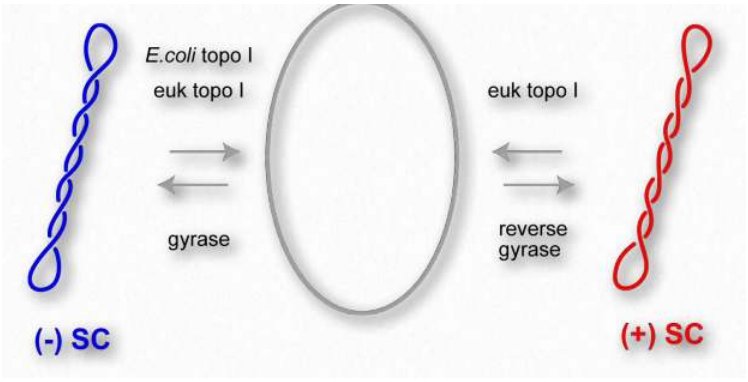


Figure 2.134 - Negatively supercoiled, relaxed circular, and positively supercoiled DNA interconverted by topoisomerase enzymes Wikipedia

Such adjustments can occur in three ways. First, tension can provide the energy for 'flipping' DNA structure. Z-DNA can arise as a means of relieving the tension. Second, DNA can 'supercoil' to relieve the tension (Figures 2.134 & 2.135). In this method, the duplex crosses over itself repeatedly, much like a rubber band will coil up if one holds one section in place and twists another part of it. Third, enzymes called topoisomerases can act to relieve or, in some cases, increase the tension by adding or removing twists in the DNA.

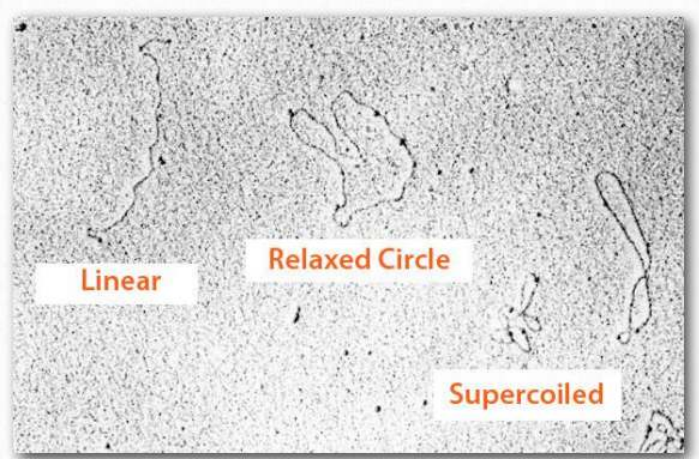


Figure 2.135 - Three topological forms of DNA Wikipedia

Topological isomers

As noted, so-called "relaxed" DNA has 10.5 base pairs per turn. Each turn corresponds to one twist of the DNA. Using enzymes, it is possible to change the number of base pairs per turn. In either the case of increasing or decreasing the twists per turn, tension is introduced into the DNA structure. If the tension cannot be relieved, the DNA duplex will act to relieve the strain, as noted. This is most easily visualized for circular DNA, though long linear DNA (such as found in eukaryotic chromosomes) or DNAs constrained in other ways will exhibit the same behavior.

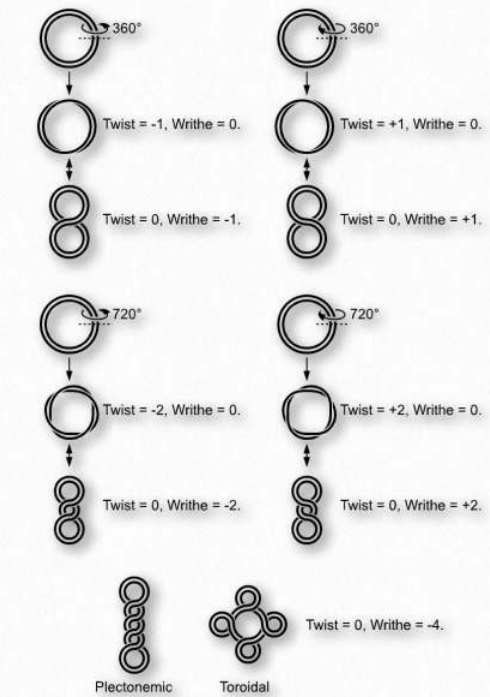


Figure 2.136 - Twists and writhes - DNA duplex coiling omitted for simplicity Wikipedia

To understand topologies, we introduce the concepts of 'writhe' and 'linking number'. First, imagine either opening a closed circle of DNA and either removing one twist or adding one twist and then re-forming the circle. Since the strands have no free ends, they cannot relieve the induced tension by re-adding or removing the twists at their ends, respectively. Instead, the tension is relieved by "superhelices" that form with crossing of the double strands over each other (figure 8 structures in Figure 2.136). Though it is not apparent to visualize, each crossing of the double strands in this way allows twists to be increased or decreased correspondingly. Thus, superhelicity allows the double helix to reassume 10.5 base pairs per turn by adding or subtracting twists as necessary and replacing them with writhes.

We write the equation L= T + W where T is the number of twists in a DNA, W is the number of writhes, and L is the linking number. The linking number is therefore the sum of the twists and writhes. Interestingly, inside of cells, DNAs typically are in a supercoiled form. Supercoiling affects the size of the DNA (compacts it) and also the expression of genes within the DNA, some having enhanced expression and some having reduced expression when supercoiling is present. Enzymes called topoisomerases alter the superhelical density of DNAs and play roles in DNA replication, transcription, and control of gene expression. They work by making cuts in one strand (Type I topoisomerases) or both strands (Type II topoisomerases) and then add or subtract twists as appropriate to the target DNA. After that process is complete, the topoisomerase re-ligates the nick/cut it had made in the DNA in the first step.

Topoisomerases may be the targets of antibiotics. The family of antibiotics known as fluoroquinolones work by interfering with the action of bacterial type II topoisomerases. Ciprofloxacin also preferentially targets bacterial type II topoisomerases. Other topoisomerase inhibitors target eukaryotic topoisomerases and are used in anti-cancer treatments.

RNA

The structure of RNA (Figure 2.137) is very similar to that of a single strand of DNA. Built of ribonucleotides, joined together by the same sort of phosphodiester bonds as in DNA, RNA uses uracil in place of thymine. In cells, RNA is assembled by RNA polymerases, which copy a DNA template in the much same way that DNA polymerases replicate a parental strand. During the synthesis of RNA, uracil is used across from an adenine in the DNA template. The building of messenger RNAs by copying a DNA template is a crucial step in the transfer of the information in DNA to a form that directs the synthesis of protein. Additionally, ribosomal and transfer RNAs serve important roles in "reading" the information in the mRNA codons and in polypeptide synthesis. RNAs are also known to play important roles in the regulation of gene expression.

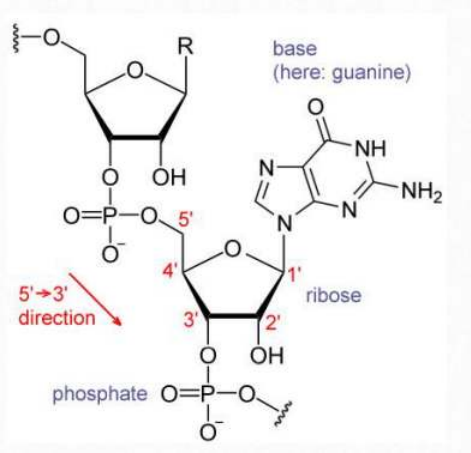


Figure 2.137 A section of an RNA molecule Wikipedia

RNA world

The discovery, in 1990, that RNAs could play a role in catalysis, a function once thought to be solely the domain of proteins, was followed by the discovery of many more so-called ribozymes- RNAs that functioned as enzymes. This suggested the answer to a long-standing chicken or egg puzzle - if DNA encodes proteins, but the replication of DNA requires proteins, how did a replicating system come into being? This problem could be solved if the first replicator was RNA, a molecule that can both encode information and carry out catalysis. This idea, called the “RNA world” hypothesis, suggests that DNA as genetic material and proteins as catalysts arose later, and eventually prevailed because of the advantages they offer. The lack of a 2’OH on deoxyribose makes DNA more stable than RNA. The double-stranded structure of DNA also provides an elegant way to easily replicate it. RNA catalysts, however, remain, as remnants of that early world. In fact, the formation of peptide bonds, essential for the synthesis of proteins, is catalyzed by RNA.

Secondary structure

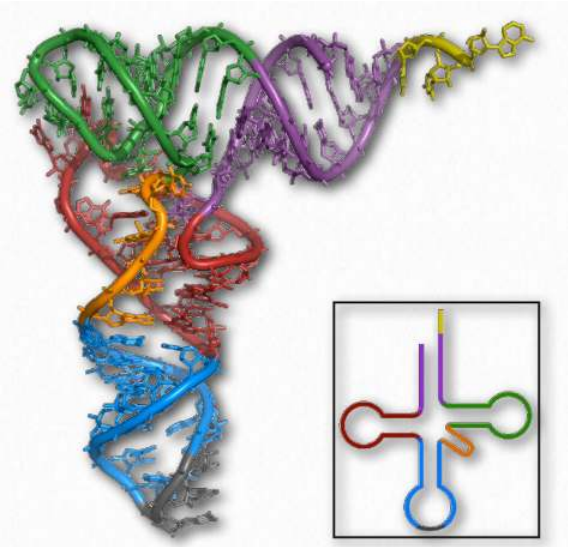


Figure 2.138 - tRNA Images - 3D projection (left) and 2D projection (inset) Wikipedia

With respect to structure, RNAs are more varied than their DNA cousin. Created by copying regions of DNA, cellular RNAs are synthesized as single strands, but they often have self-complementary regions leading to “foldbacks” containing duplex regions. These are most easily visualized in the ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) (Figure 2.138), though other RNAs, including messenger RNAs (mRNAs), small nuclear RNAs (snRNAs), microRNAs (Figure 2.139), and small interfering RNAs (siRNAs) may each have double helical regions as well.

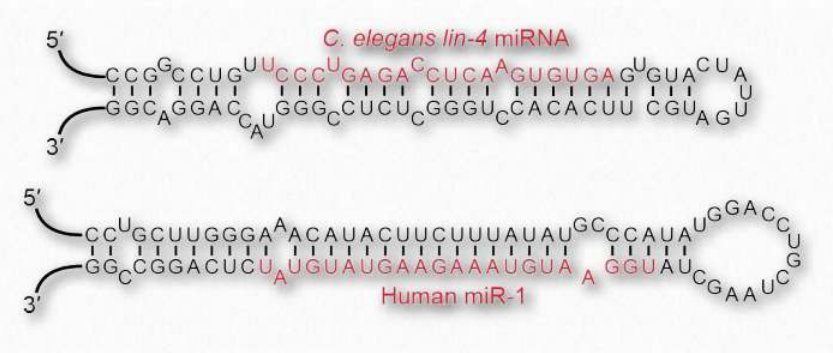


Figure 2.139 - MicroRNA stem loops. Wikipedia

Base pairing

Base pairing in RNA is slightly different than DNA. This is due to the presence of the base uracil in RNA in place of thymine in DNA. Like thymine, uracil forms base pairs with adenine, but unlike thymine, uracil can, to a limited extent, also base pair with guanine, giving rise to many more possibilities for pairing within a single strand of RNA.

These additional base pairing possibilities mean that RNA has many ways it can fold upon itself that single-stranded DNA cannot. Folding, of course, is critical for protein function, and we now know that, like proteins, some RNAs in their folded form can catalyze reactions just like enzymes. Such RNAs are referred to as ribozymes. It is for this reason scientists think that RNA was the first genetic material, because it could not only carry information, but also catalyze reactions. Such a scheme might allow certain RNAs to make copies of themselves, which would, in turn, make more copies of themselves, providing a positive selection.

Stability

RNA is less chemically stable than DNA. The presence of the 2' hydroxyl on ribose makes RNA much more prone to hydrolysis than DNA, which has a hydrogen instead of a hydroxyl. Further, RNA has uracil instead of thymine. It turns out that cytosine is the least chemically stable base in nucleic acids. It can spontaneously deaminate and in turn is converted to a uracil. This reaction occurs in both DNA and RNA, but since DNA normally has thymine instead of uracil, the presence of uracil in DNA indicates that deamination of cytosine has occurred and that the uracil needs to be replaced with a cytosine. Such an event occurring in RNA would be essentially undetectable, since uracil is a normal component of RNA. Mutations in RNA have much fewer consequences than mutations in DNA because they are not passed between cells in division.

Catalysis

RNA structure, like protein structure, has importance, in some cases, for catalytic function. Like random coils in proteins that give rise to tertiary structure, single-stranded regions of RNA that link duplex regions give these molecules a tertiary structure, as well. Catalytic RNAs, called ribozymes, catalyze important cellular reactions, including the formation of peptide bonds in ribosomes (Figure 2.114). DNA, which is usually present in cells in strictly duplex forms (no tertiary structure, per se), is not known to be involved in catalysis.

RNA structures are important for reasons other than catalysis. The 3D arrangement of tRNAs is necessary for enzymes that attach amino acids to them to do so properly. Further, small RNAs called siRNAs found in the nucleus of cells appear to play roles in both gene regulation and in cellular defenses against viruses. The key to the mechanisms of these actions is the formation of short foldback RNA structures that are recognized by cellular proteins and then chopped into smaller units. One strand is copied and used to base pair with specific mRNAs to prevent the synthesis of proteins from them.

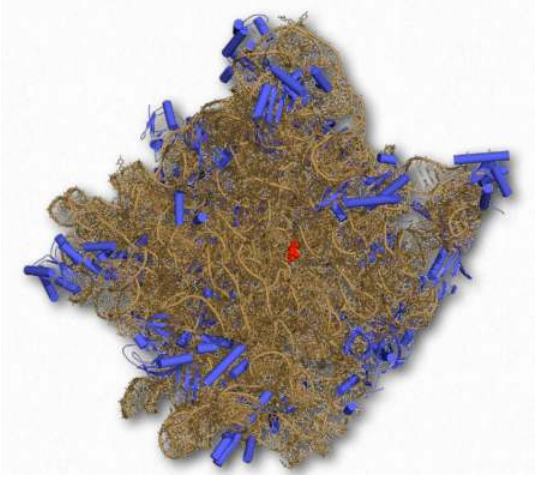


Figure 2.140 - Structure of the 50S ribosomal subunit. rRNA shown in brown. Active site in red Wikipedia

Denaturing nucleic acids

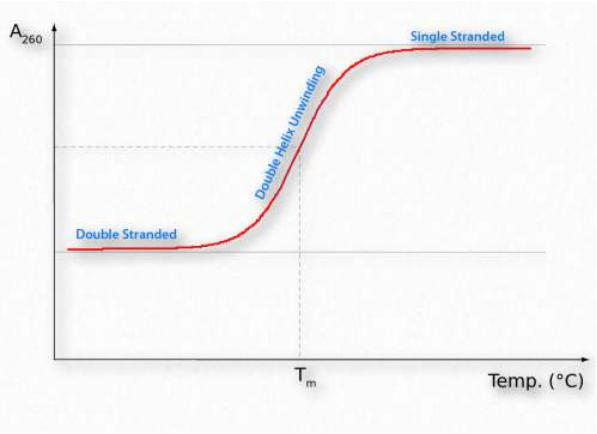


Figure 2.141 - The hyperchromic effect Wikipedia

Like proteins, nucleic acids can be denatured. Forces holding duplexes together include hydrogen bonds between the bases of each strand that, like the hydrogen bonds in proteins, can be broken with heat or urea. (Another important stabilizing force for DNA arises from the stacking interactions between the bases in a strand.) Single strands absorb light at 260 nm more strongly than double strands. This is known as the hyperchromic effect (Figure 2.141)and is a consequence of the disruption of interactions among the stacked bases. The changes in absorbance allow one to easily follow the course of DNA denaturation. Denatured duplexes can readily renature when the temperature is lowered below the “melting temperature” or Tm, the temperature at which half of the DNA strands are in duplex form. Under such conditions, the two strands can re-form hydrogen bonds between the complementary sequences, returning the duplex to its original state. For DNA, strand separation and rehybridization are important for the technique known as the polymerase chain reaction (PCR). Strand separation of DNA duplexes is accomplished in the method by heating them to boiling. Hybridization is an important aspect of the method that requires single stranded primers to “find” matching sequences on the template DNA and form a duplex. Considerations for efficient hybridization (also called annealing) include temperature, salt concentration, strand concentration, and magnesium ion levels (for more on PCR, see HERE).

DNA packaging

DNA is easily the largest macromolecule in a cell. The single chromosome in small bacterial cells, for example, can have a molecular weight of over 1 billion Daltons. If one were to take all of the DNA of human chromosomes from a single cell and lay them end to end, they would be over 7 feet long. Such an enormous molecule demands careful packaging to fit within the confines of a nucleus (eukaryotes) or a tiny cell (bacteria). The chromatin system of eukaryotes is the best known, but bacteria, too, have a system for compacting DNA.

DNA in Bacteria

In bacteria, there is no nucleus for the DNA. Instead, DNA is contained in a structure called a nucleoid (Figure 2.142). It contains about 60% DNA with much of the remainder comprised of RNAs and transcription factors. Bacteria do not have histone proteins that DNA wrap around, but they do have proteins that help organize the DNA in the cell - mostly by making looping structures.

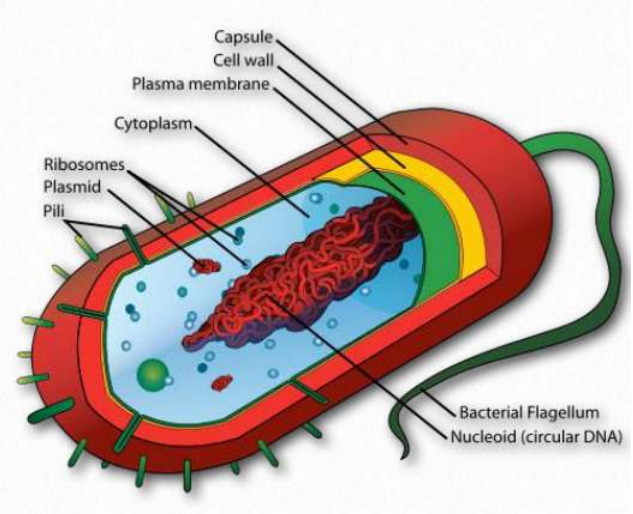


Figure 2.142 - Anatomy of a bacterial cell.

These proteins are known as Nucleoid Associated Proteins and include ones named HU, H-NS, Fis, CbpA, and Dps. Of these, HU most resembles eukaryotic histone H2B and binds to DNA non-specifically. The proteins associate with the DNA and can also cluster, which may be the origin of the loops. It is likely these proteins play a role in helping to regulate transcription and respond to DNA damage. They may also be involved in recombination.

Eukaryotes

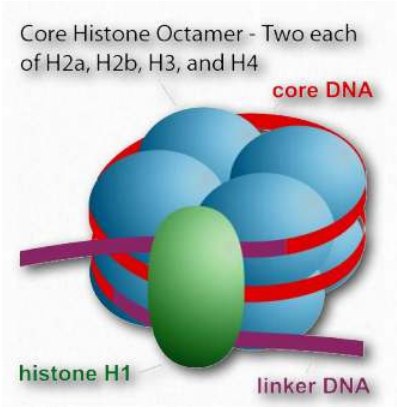


Figure 2.143 - Structure of a nucleosome Wikipedia

The method eukaryotes use for compacting DNA in the nucleus is considerably different, and with good reason - eukaryotic DNAs are typically much larger than prokaryotic DNAs, but must fit into a nucleus that is not much bigger than a prokaryotic cell. Human DNA, for example, is about 1000 times longer than c DNA. The strategy employed in eukaryotic cells is that of spooling - DNA is coiled around positively charged proteins called histones. These proteins, whose sequence is very similar in cells as diverse as yeast and humans, come in four types, dubbed H1, H2a, H2b, H3, and H4. A sixth type, referred to as H5 is actually an isoform of H1 and is rare. Two each of H2a, H2b, H3, and H4 are found in the core structure of what is called the fundamental unit of chromatin - the nucleosome (Figure 2.143).

Octamer

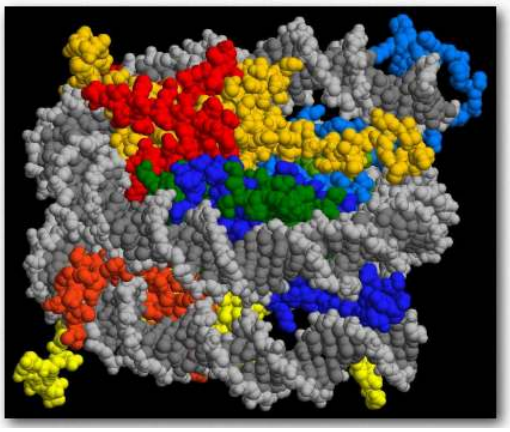


Figure 2.144 - Detailed core particle structure - DNA in gray, histones in red, yellow, green, blue Wikipedia

The core of 8 proteins is called an octamer. The stretch of DNA wrapped around the octamer totals about 147 base pairs and makes 1 2/3 turns around it. This complex is referred to as a core particle (Figure 2.144). A linker region of about 50-80 base pairs separate core particles. The term nucleosome then refers to a a core particle plus a linker region (Figure 2.143). Histone H1 sits near the junction of the incoming DNA and the histone core. It is often referred to as the linker histone. In the absence of H1, non-condensed nucleosomes resemble “beads on a string” when viewed in an electron microscope.

Histones



Figure 2.145 - Amino acid sequence (1- letter code) of histone H3 of S. cerevisiae. Arginines (R) and lysines (K) shown in red.

Histone proteins are similar in structure and are rich in basic amino acids, such as lysine and arginine (Figure 2.145). These amino acids are positively charged at physiological pH, with enables them to form tight ionic bonds with the negatively charged phosphate backbone of DNA.

For DNA, compression comes at different levels (Figure 2.146). The first level is at the nucleosomal level. Nucleosomes are stacked and coiled into higher order structures. 10 nm fibers are the simplest higher order structure (beads on a string) and they grow in complexity. 30 nm fibers consist of stacked nucleosomes and they are packed tightly. Higher level packing produces the metaphase chromosome found in meiosis and mitosis.

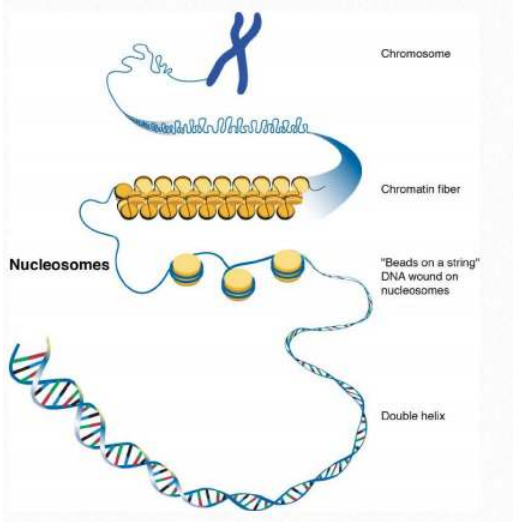


Figure 2.146 Packaging of DNA into a chromosome

https://chem.libretexts.org/@go/page/347424

The chromatin complex is a logistical concern for the processes of DNA replication and (particularly) gene expression where specific regions of DNA must be transcribed. Altering chromatin structure is therefore an essential function for transcriptional activation in eukaryotes. One strategy involves adding acetyl groups to the positively charged lysine side chains to “loosen their grip” on the negatively charged DNA, thus allowing greater access of proteins involved in activating transcription to gain access to the DNA. The mechanisms involved in eukaryotic gene expression are

Ames test

The Ames test (Figure 2.147) is an analytical method that allows one to determine whether a compound causes mutations in DNA (is mutagenic) or not. The test is named for Dr. Bruce Ames, a UC Berkeley emeritus professor who was instrumental in creating it. In the procedure, a single base pair of a selectable marker of an organism is mutated in a plasmid to render it nonfunctional. In the example, a strain of Salmonella is created that lacks the ability to grow in the absence of histidine. Without histidine, the organism will not grow, but if that one base in the plasmid’s histidine gene gets changed back to its original base, a functional gene will be made and the organism will be able to grow without histidine.

A culture of the bacterium lacking the functional gene is grown with the supply of histidine it requires. It is split into two vials. To one of the vials, a compound that one wants to test the mutagenicity of is added. To the other vial, nothing is added. The bacteria in each vial are spread onto plates lacking histidine. In the absence of mutation, no bacteria will grow. The more colonies of bacteria that grow, the more mutation happened. Note that even the vial without the possible mutagenic compound will have a few colonies grow, as a result of mutations unlinked to the potential mutagen.

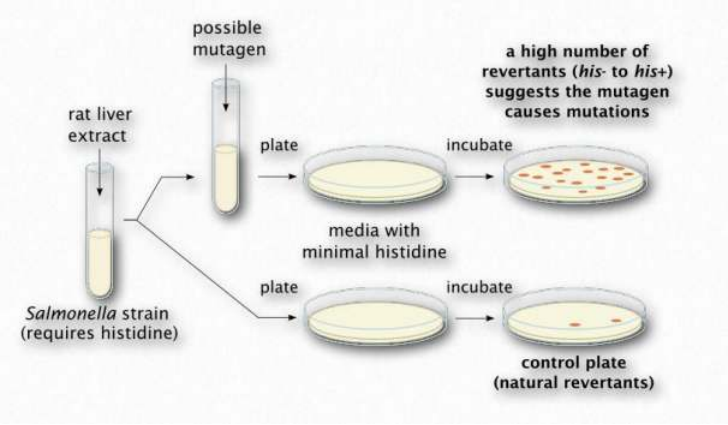


Figure 2.147 - The Ames test Wikipedia

Mutation happens in all cells at a low level. If the plate with the cells from the vial with the compound has more colonies than the cells from the control vial (no compound), then that would be evidence that the compound causes more mutations than would normally occur and it is therefore a mutagen. On the other hand, if there was no significant difference in the number of colonies on each plate, then that would suggest it is not mutagenic. The test is not perfect - it identifies about 90% of known mutagens - but its simplicity and inexpensive design make it an excellent choice for an initial screen of a compound.

This page titled 4.2: Structure and Function - Nucleic Acids is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **2.6: Structure and Function - Nucleic Acids** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

4.3: DNA Replication

Source: BiochemFFA_7_2.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy



Copying instructions

The only way to make new cells is by the division of pre-existing cells. Single-celled organisms undergo division to produce more cells like themselves, while multicellular organisms arise through division of a single cell, generally the fertilized egg. Each time a cell divides, all of its DNA must be copied faithfully so that a copy of this information can be passed on to the daughter cell. This process is called DNA replication. It is the means by which genetic information can be transmitted down generations of cells, and it ensures that every new cell has a complete copy of the genome. In the next section, we will examine the process by which the DNA of a cell is completely and accurately copied.

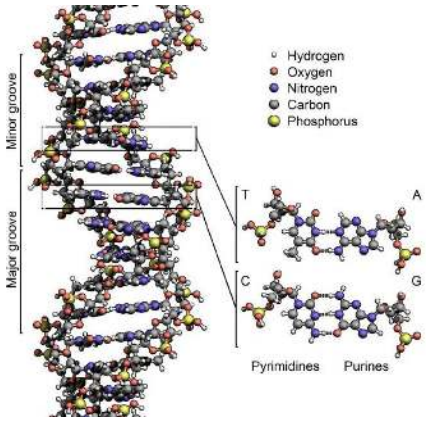


Figure 7.8 - Watson & Crick's DNA structure - Wikipedia

The structure of DNA elucidated by Watson and Crick in 1953 immediately suggested a mechanism by which double-stranded DNA could be copied to give two identical copies of the DNA. They proposed that the two strands of the DNA molecule, which are held together by hydrogen bonds between the base-paired nucleotides, would separate and each serve as a template on which a complementary strand could be assembled (Figure 7.8). The base-pairing rules would ensure that this process would result in the production of two identical DNA molecules. The beautiful simplicity of this scheme was shown to be correct in subsequent experiments by Meselson and Stahl, that demonstrated that DNA replication was semi-conservative, i.e., that after replication, each of the two resulting DNA molecules was made up of one old strand and one new strand that had been assembled across from it (Figure 7.9).

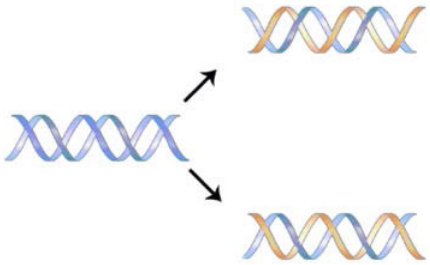


Figure 7.9 - Semi-conservative replication - Image by Martha Baker

Building materials

What are the ingredients necessary for building a new DNA molecule? As noted above, the original, or parental DNA molecule serves as the template. New DNA molecules are assembled across from each template by joining together free DNA nucleotides as directed by the base pairing rules, with As across from Ts and Gs across from Cs.

The nucleotides used in DNA synthesis are deoxyribonucleoside triphosphates or dNTPs. As can be inferred from their name, such nucleotides have a deoxyribose sugar and three phosphates, in addition to one of the four DNA bases, A, T, C or G (Figure 7.10).

When dNTPs are added into a growing DNA strand, two of those phosphates will be cleaved off, as described later, leaving the nucleotides in a DNA molecule with only one phosphate per nucleotide. This reaction is catalyzed by enzymes known as DNA polymerases, which create phosphodiester linkages between one nucleotide and the next.

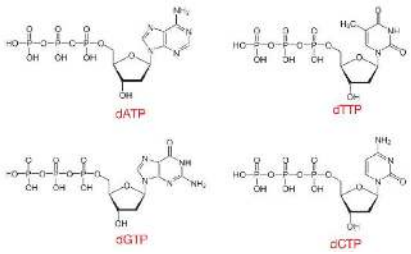


Figure 7.10 - The building blocks of DNA

Challenges

Before examining the actual process of DNA replication, it is useful to think about what it takes to accomplish this task successfully. Consider the challenges facing a cell in this process:

- The sheer number of nucleotides to be copied is enormous: e.g., in human cells, on the order of several billions.
- A double-helical parental DNA molecule must be unwound to expose single strands of DNA that can serve as templates for the synthesis of new DNA strands.
- Unwinding must be accomplished without introducing topological distortion into the molecule.
- The unwound single strands of DNA must be kept from coming back together long enough for the new strands to be synthesized.
- DNA polymerases cannot begin synthesis of a new DNA strand de novo and require a free 3' OH to which they can add deoxynucleotides.
- DNA polymerases can only extend a strand in the 5' to 3' direction. The 5' to 3' growth of both new strands means that one of the strands is made in pieces.
- The use of RNA primers requires that the RNA nucleotides must be removed and replaced with DNA nucleotides and the resulting DNA fragments must be joined.
- The copying of all the parental DNA must be accurate, so that mutations are not introduced into the newly made DNA.

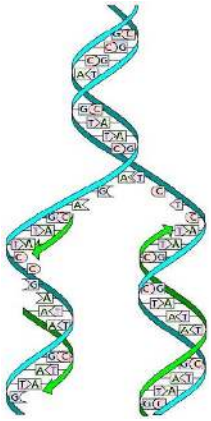


Figure 7.11 - Replication of DNA - Wikipedia



Figure 7.12 - Human chromosomes

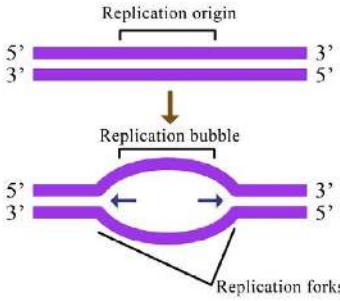


Figure 7.13 - Bidirectional replication from an origin - Martha Baker

Prokaryotic DNA Replication	Eukaryotic DNA replication
Occurs inside the cytoplasm	Occurs inside the nucleus
Only one origin of replication per molecule of DNA	Have many origins of replication in each chromosome
Origin of replication is about 100-200 or more nucleotides in length	Each origin of replication is formed of about 150 nucleotides
Replicaton occurs at one point in each chromosome	Replication occurs at several points simultaneously in each chromosome
Only have one origin of replication	Has multiple origins of replication
Initiation is carried out by protein DnaA and DnaB	Initiation is carried out by the Origin Recognition Complex
Topoisomerase is needed	Topoisomerase is needed
Replicaton is very rapid	Replication is slow

Figure 7.14 - Prokaryotic vs. eukaryotic DNA replication - Wikipedia

Addressing challenges

With this in mind, we can begin to examine how cells deal with each of these challenges. Our understanding of the process of DNA replication is derived from studies using bacteria, yeast, and other systems. These investigations have revealed that DNA replication is carried out by the action of a large number of proteins that act together as a complex protein machine. Numerous proteins involved in replication have been identified and characterized, including multiple different DNA polymerases in both

prokaryotes and eukaryotes. Although the specific proteins involved are different in bacteria and eukaryotes, it is useful to understand the basic considerations that are relevant in all cells. A generalized account of the steps in DNA replication is presented below, focused on the challenges mentioned above.

- The sheer number of nucleotides to be copied is enormous: e.g., in human cells, on the order of several billions.

Cells, whether bacterial or eukaryotic, have to replicate all of their DNA before they can divide. In cells like our own, the vast amount of DNA is broken up into many chromosomes, each of which is composed of a linear strand of DNA (Figure 7.12). In cells like those of E. coli, there is a single circular chromosome.

In either situation, DNA replication is initiated at sites called origins of replication. These are regions of the DNA molecule that are recognized by special proteins called initiator proteins that bind the DNA. In E.coli, origins have small regions of A-T-rich sequences that are “melted” to separate the strands, when the initiator proteins bind to the origin or replication. As you may remember, A-T base-pairs, which have two hydrogen bonds between them are more readily disrupted than G-C base-pairs which have three apiece (Figure 7.15).

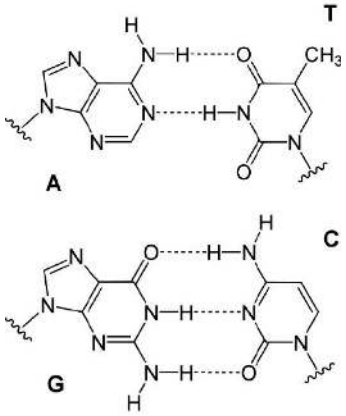


Figure 7.15 - A-T and G-C base pairs

How many origins of replication are there on a chromosome? In the case of E. coli, there is a single origin of replication on its circular chromosome. In eukaryotic cells there may be many thousands of origins of replication, with each chromosome having hundreds (Figure 7.16). DNA replication is, thus, initiated at multiple points along each chromosome in eukaryotes. Electron micrographs of replicating DNA from eukaryotic cells show many replication bubbles on a single chromosome. This makes sense in light of the large amount of DNA that there is to be copied in cells like our own, where beginning at one end of each chromosome and replicating all the way through to the other end from a single origin would simply take too long. This is despite the fact that the DNA polymerases in human cells are capable of building new DNA strands at the very respectable rate of about 50 nucleotides per second!

- A double-helical parental molecule must be unwound to expose single strands of DNA that can serve as templates for the synthesis of new DNA strands.

Unwinding

Once a small region of the DNA is opened up at each origin of replication, the DNA helix must be unwound to allow replication to proceed. The unwinding of the DNA helix requires the action of an enzyme called helicase.

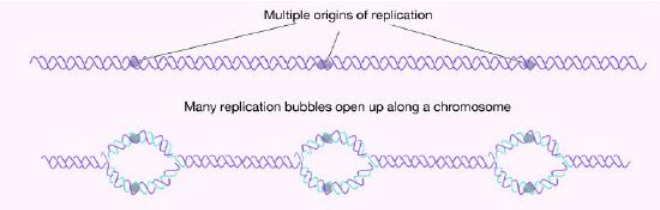


Figure 7.16 - Multiple replication bubble on a eukaryotic chromosome - Image by Martha Baker

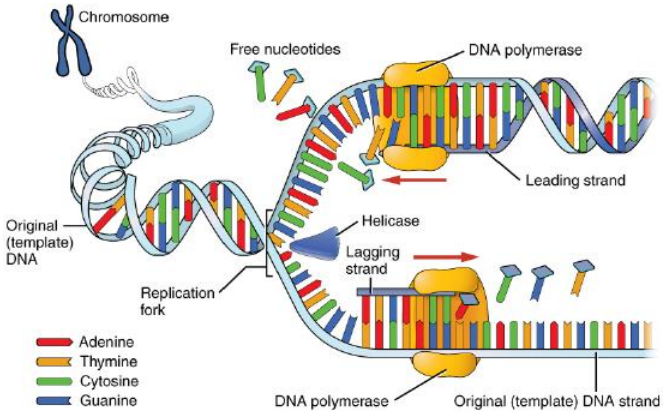


Figure 7.17 - View of a eukaryotic DNA replication fork with helicase shown in blue - Wikipedia

Helicase uses the energy released when ATP is hydrolyzed, to break the hydrogen bonds between the bases in DNA and separate the two strands (Figure 7.17). Note that a replication bubble is made up of two replication forks that "move" or open up, in opposite directions. At each replication fork, the parental DNA strands must be unwound to expose new sections of single-stranded template.

- This unwinding must be accomplished without introducing topological distortion into the molecule.

What is the effect of unwinding one region of the double helix? Local unwinding of the double helix causes over-winding (increased positive supercoiling) ahead of the unwound region.

The DNA ahead of the replication fork has to rotate, or it will get twisted on itself and halt replication. This is a major problem, not only for circular bacterial chromosomes, but also for linear eukaryotic chromosomes, which, in principle, could rotate to relieve the stress caused by the increased supercoiling.

Topoisomerases

The reason this is problematic is that it is not possible to rotate the entire length of a chromosome, with its millions of base-pairs, as the DNA at the replication fork is unwound. How, then, is this problem solved? Enzymes called topoisomerases can relieve the topological stress caused by local “unwinding” of the extra winds of the double helix. They do this by cutting one or both strands of the DNA and allowing the strands to swivel around each other to release the tension before rejoining the ends. In E. coli, the topoisomerase that performs this function is called gyrase.

- The separated single strands of DNA must be kept from coming back together so the new strands to be synthesized.

Single-strand DNA binding protein

Once the two strands of the parental DNA molecule are separated, they must be prevented from going back together to form double-stranded DNA. To ensure that unwound regions of the parental DNA remain single-stranded and available for copying, the separated strands of the parental DNA are bound by many molecules of a protein called single-strand DNA binding protein (SSB - Figure 7.18).

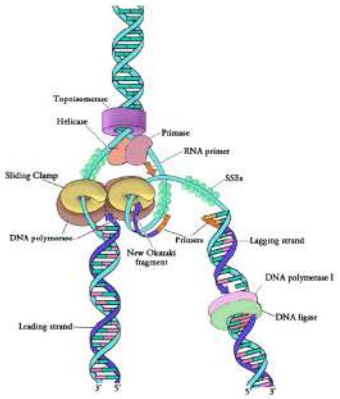


Figure 7.18 - Proteins at a prokaryotic DNA replication fork - Image by Martha Baker

- DNA polymerases cannot begin synthesis of a new DNA strand de novo and require a free 3' OH to which they can add DNA nucleotides.

Although single-stranded parental DNA is now available for copying, DNA polymerases cannot begin synthesis of a complementary strand de novo. This simply means that DNA polymerases can only add new nucleotides on to the 3' end of a pre-existing chain, and cannot start a chain of nucleotides on their own. Because of this limitation, some enzyme other than a DNA polymerase must first make a small region of nucleic acid, complementary to the parental strand, that can provide a free 3' OH to which DNA polymerase can add a deoxyribonucleotide. This task is accomplished by an enzyme called a **primase**, which assembles a short stretch of RNA base-paired to the parental DNA template. This provides a short base-paired region, called the RNA primer, with a free 3'OH group to which DNA polymerase can add the first new DNA nucleotide (Figure 7.12).

Sliding clamp

Once a primer provides a free 3'OH for extension, other proteins get into the act. These proteins are involved in loading the DNA polymerase onto the primed template and keeping it associated with the DNA. The first of these is the clamp loader. As its name suggests, the clamp loader helps to load a protein complex called the sliding clamp onto the DNA at the replication fork (Figure 7.19 and 7.20). The sliding clamp, a multi-subunit ring-shaped protein, is then joined by the DNA Polymerase. The function of the sliding clamp is to keep the polymerase associated with the replication fork - in fact, it has been described as a seat-belt for the DNA polymerase. The sliding clamp ensures that the DNA polymerase is able to synthesize long stretches of new DNA before it dissociates from the template. The property of staying associated with the template for a long time before dissociating is known as the processivity of the enzyme. In the presence of the sliding clamp, DNA polymerases are much more processive, making replication faster and more efficient.

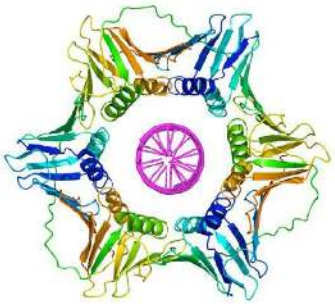


Figure 7.19 - Top view of the sliding clamp (outside) surrounding DNA strand (inside)

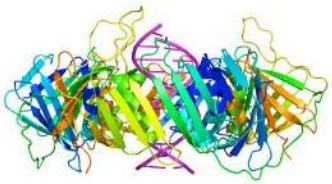


Figure 7.20 - Side view of the sliding clamp surrounding a DNA strand (in purple)

Extending the primer

The DNA polymerase is now poised to start synthesis of the new DNA strand (in E. coli, the primary replicative polymerase is called DNA polymerase III). As you already know, the synthesis of new DNA is accomplished by the addition of new nucleotides complementary to those on the parental strand. DNA polymerase catalyzes the reaction by which an incoming deoxyribonucleotide, complementary to the template, is added onto the 3' end of the previous nucleotide, starting with the 3'OH on the end of the RNA primer. The importance of the 3’OH group lies in the nature of the reaction that builds a chain of nucleotides.

The reaction catalyzed by the DNA polymerase occurs through the nucleophilic attack by the 3’OH group at the end of a nucleic acid strand on the α phosphate of the incoming dNTP (Figure 7.21). The immediate hydrolysis of the pyrophosphate that is cleaved

off the incoming dNTP drives the reaction forward. The sequential addition of new nucleotides at the 3’ end of the growing chain of DNA accounts for the fact that the strand grows in a 5’ to 3’ direction.

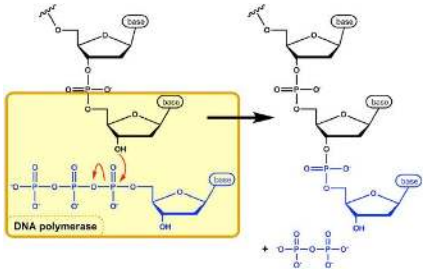


Figure 7.21 - Mechanism of DNA chain growth - Wikipedia

The 5' phosphate on each incoming nucleotide is joined by the DNA polymerase to the 3' OH on the end of the growing nucleic acid chain, to make a phosphodiester bond. Each added nucleotide provides a new 3’OH, allowing the chain to be extended for as long as the DNA polymerase continues to synthesize the new strand. As we already noted, the new DNA strands are synthesized by the addition of DNA nucleotides to the end of an RNA primer. The new DNA molecule thus has a short piece of RNA at the beginning.

- DNA polymerases can only extend a strand in the 5' to 3' direction. The 5' to 3' growth of both new strands means that one of the strands is made in pieces.

Leading strand

We know that DNA polymerases can only build a new DNA strand in the 5' to 3' direction. We also know that the two parental strands of DNA are antiparallel. This means that at each replication fork, one new strand, called the leading strand can be synthesized continuously in the 5' to 3' direction because it is being made in the same direction that the replication fork is opening up.

Lagging strand

The synthesis of the other new strand, called the lagging strand, also proceeds in the 5’ to 3’ direction. But because the template strands are running in opposite directions, the lagging strand is being extended in the direction opposite to the opening of the replication fork (Figure 7.22). As the replication fork opens up, the region behind the original start point for the lagging strand will need to be copied. This means another RNA primer must be laid down and extended. This process repeats itself as the replication fork opens up, with multiple RNA primers laid down and extended, producing many short pieces that are later joined. These short nucleic acid pieces, each composed of a small stretch of RNA primer and about 1000-2000 DNA nucleotides, are called Okazaki fragments, for Reiji Okazaki, the scientist who first demonstrated their existence.

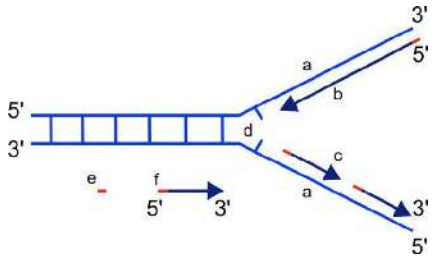


Figure 7.22 - Bidirectional DNA replication with template strand (a), leading strand (b), Okazaki fragments (c), replication fork (d), RNA primer (e), and DNA extended RNA primers (f) - Wikipedia

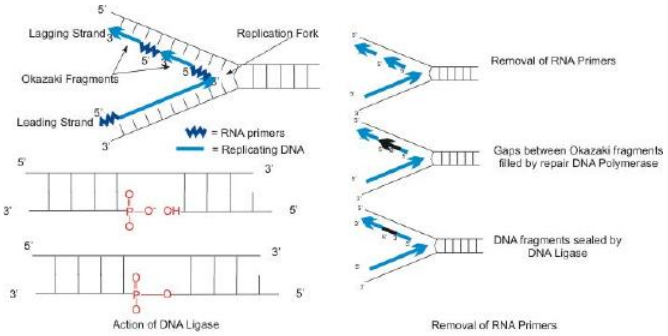


Figure 7.23 - Removal of RNA primers and joining of Okazaki fragments - Wikipedia

- The use of RNA primers requires that the RNA nucleotides must be removed and replaced with DNA nucleotides.

Primer removal

We have seen that each newly synthesized piece of DNA starts out with an RNA primer, effectively making a new nucleic acid strand that is part RNA and part DNA. The newly made DNA strand cannot be allowed to have pieces of RNA attached. So, the RNA nucleotides must be removed and the gaps filled in with DNA nucleotides (Figure 7.23). This is done by DNA polymerase I in E. coli. This enzyme begins adding DNA nucleotides at the end of each Okazaki fragment. However, the end of one Okazaki fragment is adjacent to the RNA primer at the beginning of the next Okazaki fragment. DNA polymerase I has an exonuclease activity acting in the 5' to 3' direction that removes the RNA nucleotides ahead of it, while the polymerase activity replaces the RNA nucleotides with dNTPs. Once all the RNA nucleotides have been removed, the lagging strand is made up of stretches of DNA. The DNA pieces are then joined together by the enzyme DNA ligase.

The steps outlined above essentially complete the process of DNA replication. But one issue still remains.

- Ensuring accuracy in the copying of so much information

Accuracy

How accurate is the copying of information by DNA polymerase? As you are aware, changes in DNA sequence (mutations) can change the amino acid sequence of the encoded proteins and that this is often, though not always, deleterious to the functioning of the organism. When billions of bases in DNA are copied during replication, how do cells ensure that the newly synthesized DNA is a faithful copy of the original information?

DNA polymerases, as we have noted earlier work fast (averaging 50 bases a second in human cells and up to 200 times faster in E. coli). Yet, both human and bacterial cells seem to replicate their DNA quite accurately. This is because replicative DNA polymerases have a proofreading function that enables the polymerase to detect when the wrong base has been inserted across from a template strand, back up and remove the mistakenly inserted base, before continuing with synthesis (Figure 7.24).

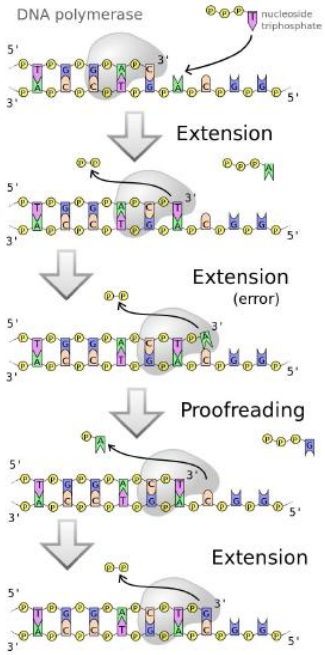


Figure 7.24 - Error corrected by DNA polymerases

Multiple activities

This is possible because most DNA polymerases are dual-function enzymes. They can extend a DNA chain by virtue of their 5' to 3' polymerase activity. Some polymerases like DNA polymerase I can also remove RNA primers in the 5’ to 3’ direction, though that is not a common activity of polymerases. Many polymerases, however have an ability to backtrack and remove the last inserted base because they possess a 3' to 5' exonuclease activity.

The exonuclease activity of a DNA polymerase allows it to excise a wrongly inserted base, after which the polymerase activity inserts the correct base and proceeds with extending the strand.

In other words, the DNA polymerase is monitoring its own accuracy (also termed its fidelity) as it makes new DNA, correcting mistakes immediately before moving on to add the next base. This mechanism, which operates during DNA replication, corrects many errors as they occur, reducing by about a 100-fold the mistakes made when DNA is copied.

DNA polymerases

As noted earlier, both prokaryotic and eukaryotic cells have multiple DNA polymerases. In E.coli, for example, DNA polymerase III is the major replicative polymerase (a.k.a. replicase) while DNA polymerase I is responsible for DNA repair as well as removal of RNA primers and their replacement with DNA nucleotides during replication. DNA polymerase II plays a role in restarting replication after DNA damage stalls replication, while DNA polymerases IV and V are both required in trans-lesion, or bypass, synthesis, which allows replication past sites of DNA damage.

Eukaryotic polymerases

In eukaryotes, there are over fifteen different DNA polymerases. The primary replicative polymerases in the nucleus are ∂ and ε. DNA polymerase α is also important for replication because it has primase and repair activities. Replication is initiated in eukaryotic cells by DNA polymerase α, which binds to the initiation complex at the origin and lays down an RNA primer, followed by about 25 nucleotides of DNA. It is then replaced by another polymerase, in a step called the pol switch. DNA polymerase ∂ or ε then continues synthesizing DNA, depending on the strand. The role of polymerase ε appears to be synthesis of the leading strand due to its high processivity and accuracy, whereas polymerase ∂ extends Okazaki fragments on the lagging strands. Proteins analogous to the clamp loader and sliding clamp are also present. The protein RFC plays the role of clamp loader, while another protein, PCNA acts like the sliding clamp. Several other DNA polymerases like β, γ and μ function in repairing gaps. Yet others are involved in trans-lesion synthesis following DNA damage and are associated with hypermutation.

Despite their diversity, DNA polymerases share some common structural features. X-ray crystallographic studies have shown that these enzymes have a structure that has been compared to a human right hand (Figures 7.25 & 7.26). The «palm» of the hand forms a cleft in which the DNA lies. The cleft is also the where the polymerase catalytic activity resides. This is where the incoming nucleotide is added on to the growing chain. «The fingers» position the DNA in the active site, while the «thumb» holds the DNA as it exits the polymerase. A separate domain contains the exonuclease (proofreading) activity of the enzyme.

The enzyme alternates between its polymerizing activity and its proofreading activity. When a mismatched base pair is in the polymerase catalytic site, the 3'end of the growing strand is moved from the polymerase site to the exonuclease active site (Figure 7.26). The mispair at the end is removed by the exonuclease, followed by repositioning of the 3' end in the polymerase active site to continue synthesis.

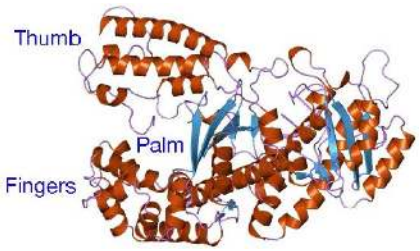


Figure 7.25 DNA polymerase I with hand structure

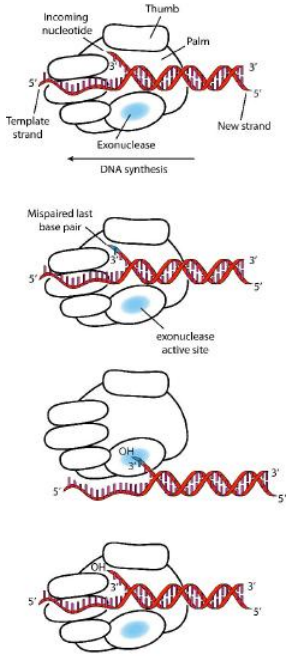


Figure 7.26 - Proofreading by DNA polymerase -Image by Pehr Jacobson

Name	Name	M.W.	Other Activity	Function
α	alpha	165,000	Primase	Repair
β	beta	39,000	Lyase	Base excision repair
δ	delta	100,000	3' exonuclease	Replication, DNA repair
ε	epsilon	225,000	3' exonuclease	Replication, DNA repair
γ	gamma	140,000	3' exonuclease	Mitochondrial replication
η	eta	78,000		Bypass of thymine dimers
ι	iota	80,000	Lyase	Repair?
K	kappa	76,000		Nucleotide excision repair
λ	lambda	66,000	Lyase	Repair/recombination
μ	mu	55,000		Repair/recombination
ν	nu	100,000		Repair?
θ	theta	290,000	Helicase	Repair? Hypermutation?
ζ	zeta	353,000		Repair/hypermutation

Figure 7.27 - Mammalian DNA polymerases

Termination of replication

In circular bacterial chromosomes, there are specific sequences known as terminator or Ter sites. These are multiple short sequences that serve as termination sites, allowing the replication forks traveling clockwise and anticlockwise across the circular chromosome to meet at one of the sites.

The binding of a protein, Tus, at a Ter site prevents further movement of the replication fork and ends replication. The parental and newly made circular DNA are, at this point topologically interlinked and must be separated with the help of topoisomerase.

The end-replication problem

There is no fixed site for termination in linear eukaryotic chromosomes. As the replication forks reach the ends of the chromosome, the leading strand can be synthesized all the way to the end of the template strand. On the lagging strand, the need for an RNA primer to start synthesis creates a challenge. When the RNA primer at the extreme end of the lagging strand is removed, there is a small stretch of the template strand that cannot be copied. As a result, in each round of replication a short sequence at the ends of the chromosome will be lost. Over time, with many cycles of replication, chromosomes would become noticeably shorter. This shortening of chromosomes has been observed in vitro, in cultured mammalian somatic cells. It is also seen in intact organisms, with increasing age.

Telomeres

What effect does the loss of sequence from the ends of the chromosomes have on cells? We know that the ends of chromosomes are characterized by structures called telomeres (Figure 7.28). Telomeres are made up of many copies of short repeated sequences (in humans, the repeat is TTAGGG) and special proteins that specifically bind to these sequences. This structure of telomeres is useful in distinguishing the ends of chromosomes from double-strand breaks in DNA, thus preventing the DNA repair mechanisms in cells from joining chromosomes end to end.

The other advantage of the repeated sequences, which do not encode proteins, is that losing some of the repeats does not lead to loss of important coding information. Thus, the repeats act as a sort of buffer zone, where the loss of sequence does not doom the cell. However, the shortening of chromosomes cannot continue indefinitely. After a certain number of replication cycles, cells are known to stop dividing and enter a state known as replicative senescence. This suggests that the shortening of the telomeres serves as a sort of clock, with the extent of shrinkage of the chromosomes serving as a measure of aging. Eventually cells that enter senescence will die.

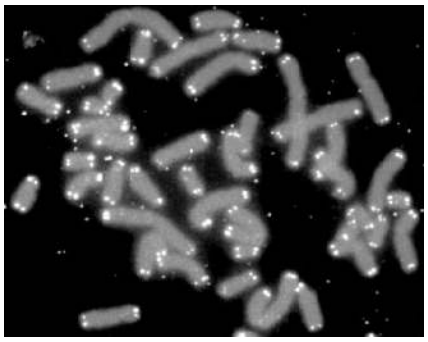


Figure 7.28 - Chromosomes with telomeres marked in white

Problems with sequence loss

Even if our cells are able to function with shorter chromosomes during our lifetimes, this leaves us with another problem. If our chromosomes grow shorter with age, then presumably our children, who inherit our chromosomes will be born with shorter chromosomes than we started with. They, in turn, would have their chromosomes shrink as they grew older, and their children would have even shorter chromosomes. Over the course of multiple generations, this would lead to the point where further chromosome shrinkage would result in cells that would enter senescence very early in life and die soon after. This obviously does not happen. Generation after generation of children are born with full-length chromosomes, so there is a mechanism that must ensure that at least in the reproductive cells, chromosomes do not get shorter.

To understand this mechanism, it is necessary to first examine the end of a newly made DNA molecule (Figure 7.29). While the leading strand, which grows in the same direction as the movement of the replication fork, can copy its template all the way to the end, the lagging strand encounters a problem. RNA primers are, as we noted, needed to start each of the Okazaki fragments of the lagging strand. The primers must be removed later, and the RNA nucleotides replaced with DNA nucleotides. When the RNA primer across from the end of the parental strand is removed, the RNA nucleotides cannot be replaced by DNA nucleotides because the DNA polymerase has no primer to start from. A short region of the template cannot, therefore be copied.

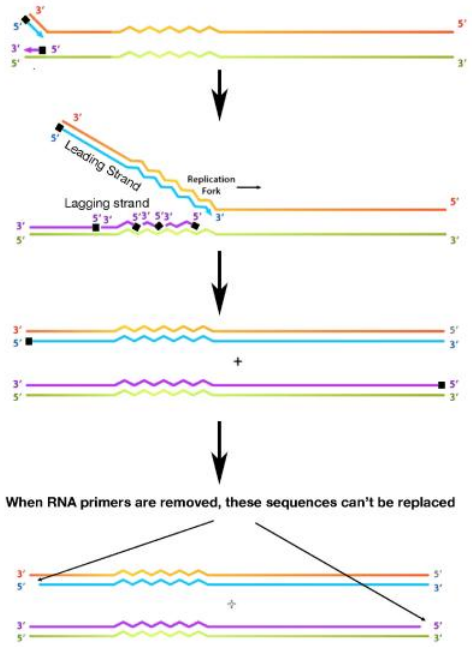


Figure 7.29 - Replication of a linear chromosome results in loss of sequences at the very ends with each round of replication

Telomerase

How can this problem be solved? It can be seen from Figure 7.29 that the end of the original template strand has a short 3' overhang resulting from the removal of the RNA primer across from it. In order to fill in this region, another primer would be needed, situated past the end of the template strand. But in order to build such a primer, it would be necessary for the template overhang to be longer. If it were possible to make the template strand longer, then another primer could be placed across from its end and the end of the strand could be copied. Such an extension of the template strand is exactly what happens in our reproductive cells. The parental template strand is extended by the enzyme telomerase, which adds telomere repeats and lengthens the template. We will see shortly how it accomplishes that feat.

RNA template

Telomerase is an unusual enzyme, in that it is made up of two components, an RNA and a reverse transcriptase. A reverse transcriptase is an RNA-dependent DNA polymerase, an enzyme that copies an RNA template to make DNA. The RNA component of the human telomerase, called hTERC, has a sequence that is complementary to the telomere repeat, TAGGG. As seen in Figure 7.31, this RNA can base-pair with the last telomere repeat on the parental DNA strand, while a portion of the RNA remains unpaired.



Figure 7.30 - Telomerase holding its RNA template (in center) - Wikipedia

Template for extension

The function of the unpaired region of the RNA is to serve as a template that can be used to extend the overhanging 3' end of the original DNA molecule. The protein component of telomerase has reverse transcriptase activity and can copy the RNA sequence into DNA. In human telomerase, the protein component is known as hTERT (telomerase reverse transcriptase). As seen in Figure 7.31 and 7.32, the reverse transcriptase extends the original 3' overhang using the RNA component as its template. The telomerase can then dissociate, and repeat the process multiple times to add many repeats of the telomere sequence.

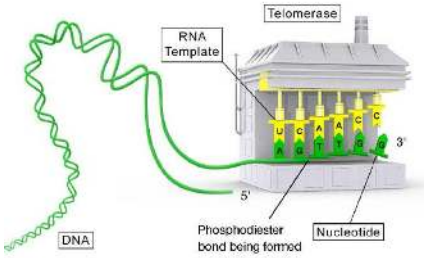


Figure 7.31 - Telomerase extends the template strand using its own RNA as template - Wikipedia

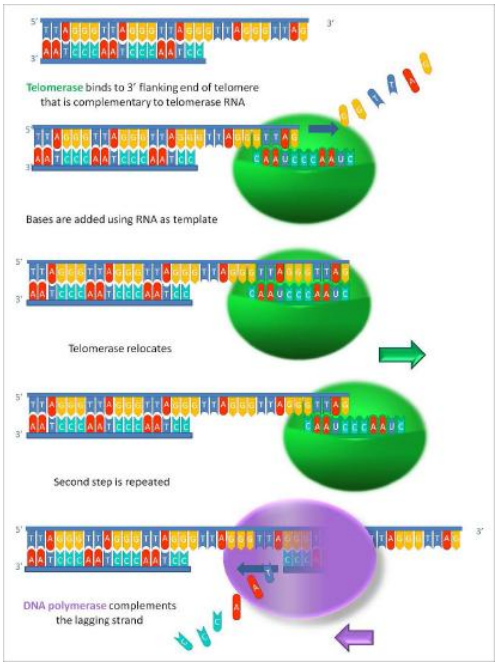


Figure 7.32 - Growing telomeres with telomerase and DNA polymerase. First, the 3' end of the template is extended by telomerase, then DNA polymerase completes synthesis of the lagging strand

Once the overhang has been extended by the addition of at least several telomere repeats, there is now room for the synthesis of an RNA primer complementary to the newly extended overhang (pointing back towards the rest of the chromosome). This primer can then be extended to complete synthesis of the lagging strand all the way to the end of the original parental DNA strand. Thus, the addition of telomere repeats on the parental DNA strands keeps the newly made DNA strands from becoming shorter with each cycle of replication. The fact that this happens in germ cells (reproductive cells) explains why each generation does not have shorter chromosomes than the parental generation.

The proofreading function of DNA polymerases monitors the accuracy of DNA replication while the enzyme telomerase keeps chromosomes that will be passed on to offspring from shortening. Between them, these two activities ensure that the genetic information is copied accurately, and that succeeding generations receive a full complement of the genetic information

Disassembly and reassembly of nucleosomes

The events of replication have an additional twist in eukaryotes. Recall that DNA is found in eukaryotic cells as chromatin, a complex of the DNA with proteins. At its least condensed, chromatin looks like a string of beads, consisting of the DNA wrapped

around histone cores to make nucleosomes. The nucleosome structure must be disrupted to make DNA available for replication and restored after replication is completed (Figure 7.33).

Ahead of the replication fork, chromatin structure is disassembled by ATP-dependent chromatin remodeling complexes, allowing access to the DNA template. Once the new strands of DNA have been synthesized, both the original nucleosomes and new nucleosomes must be reassembled behind the replication fork. Since replication gives rise to two DNA molecules where there was one, twice the amount of histones is needed to package the DNA. Preparation for DNA replication, therefore, involves the synthesis of large amounts of histones to supply the need. Interestingly, it appears that newly synthesized DNA is packaged into nucleosomes using the original histones that were displaced to allow the replication fork to pass, as well as newly synthesized histones.

We also know that post-translational modifications like acetylation, methylation or phosphorylation of the histones can regulate the degree to which a given region of the genome is accessible for use. One question that remains the subject of intense research is how these modifications are accurately passed on to the new nucleosomes.

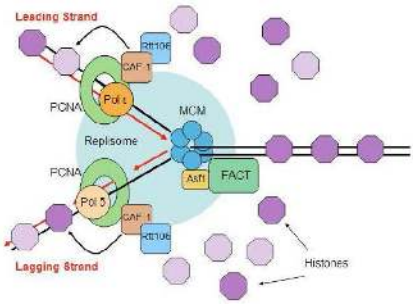


Figure 7.33 - Disruption of nucleosomes during DNA replication - Wikipedia

---

This page titled 4.3: DNA Replication is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **7.3: DNA Replication** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

4.4: DNA Repair

Source: BiochemFFA_7_3.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy



Safeguarding the genome

In the last section we considered the ways in which cells deal with the challenges associated with replicating their DNA, a vital process for all cells. It is evident that if DNA is the master copy of instructions for an organism, then it is important not to make mistakes when copying the DNA to pass on to new cells. Although proofreading by DNA polymerases greatly increases the accuracy of replication, there are additional mechanisms in cells to further ensure that newly replicated DNA is a faithful copy of the original, and also to repair damage to DNA during the normal life of a cell.

DNA damage

All DNA suffers damage over time, from exposure to ultraviolet and other radiation, as well as from various chemicals in the environment (Figures 7.34 & 7.35). Even chemical reactions naturally occurring within cells can give rise to compounds that can damage DNA. As you already know, even minor changes in DNA sequence, such as point mutations can sometimes have far-reaching consequences. Likewise, unrepaired damage caused by radiation, environmental chemicals or even normal cellular chemistry can interfere with the accurate transmission of information in DNA. Maintaining the integrity of the cell's "blueprint" is of vital importance and this is reflected in the numerous mechanisms that exist to repair mistakes and damage in DNA.

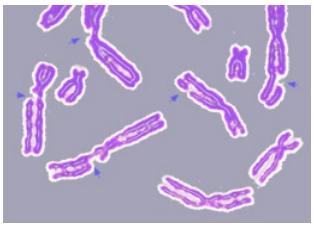


Figure 7.34 - Chromosome breaks - Wikipedia

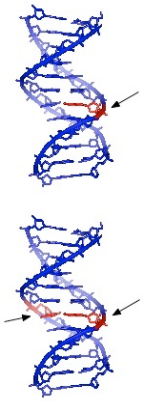


Figure 7.35 - Single- (top) and double-strand (bottom) damaged DNA - Wikipedia

Post-replicative mismatch repair

We earlier discussed proofreading by DNA polymerases during replication. While proofreading significantly reduces the error rate, not all mistakes are fixed on the fly by DNA polymerases.

What mechanisms exist to correct the replication errors that are missed by the proof-reading function of DNA polymerases? Errors that slip by proofreading during replication can be corrected by a mechanism called mismatch repair. While the error rate of DNA replication is about one in 107 nucleotides in the absence of mismatch repair, this is further reduced a hundred-fold to one in 109 nucleotides when mismatch repair is functional.

What are the tasks that a mismatch repair system faces?

It must:

- Scan newly made DNA to see if there are any mispaired bases (e.g., a G paired to a T)
- Identify and remove the region of the mismatch.
- Correctly fill in the gap created by the excision of the mismatch region.

Distinguishing strands

Importantly, the mismatch repair system must have a means to distinguish the newly made DNA strand from the template strand, if replication errors are to be fixed correctly. In other words, when the mismatch repair system encounters an A-G mispair, for example, it must know whether the A should be removed and replaced with a C or if the G should be removed and replaced with a T.

But how does the mismatch repair system distinguish between the original and the new strands of DNA? In bacteria, the existence of a system that methylates the DNA at GATC sequences is the solution to this problem. E.coli has an enzyme, DNA adenine methylase (Dam) that adds methyl groups on the to adenines in GATC sequences in DNA (Figure 7.36). Newly replicated DNA has not yet undergone methylation and thus, can be distinguished from the template strand, which is methylated.

The mismatch repair proteins selectively replace the strand lacking methylation, thus ensuring that it is mistakes in the newly made strand that are removed and replaced. Because methylation is the criterion that enables the mismatch repair system to choose the strand that is repaired, the bacterial mismatch repair system is described as being methyl-directed.

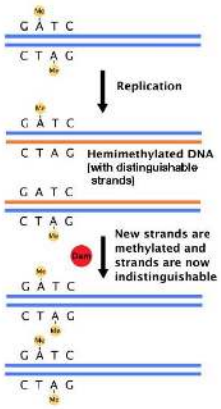


Figure 7.36- Dam methylase adds methyl groups at GATC sequences

Mismatch repair genes

Mismatch repair has been well studied in bacteria, and the proteins involved have been identified. In E.coli, mismatch repair proteins are encoded by a group of genes collectively known as the mut genes. Important components of the mismatch repair machinery are the proteins MutS, MutL and MutH (Figure 7.37).

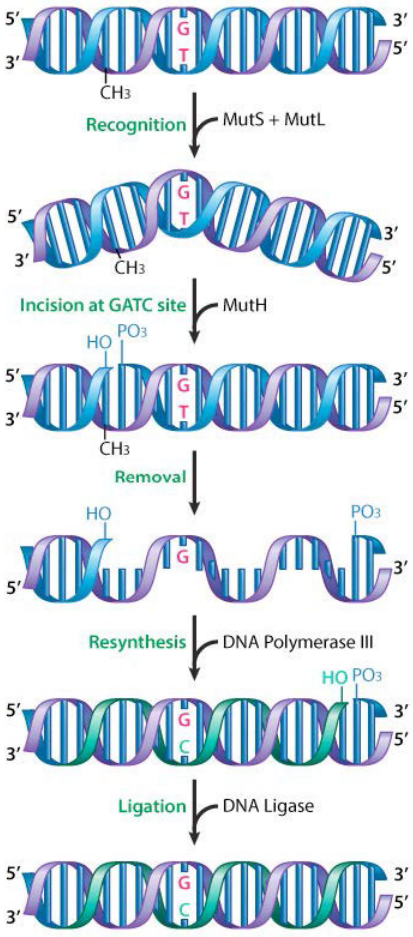


Figure 7.37 - Mismatch repair in E. coli - Image by Aleia Kim

MutS acts to recognize the mismatch, while MutL and MutH are recruited to the mismatch site by the binding of MutS. MutH is an endonuclease that cuts the newly synthesized and, as yet, unmethylated DNA strand at a GATC. This activates a DNA helicase and

an exonuclease that help unwind and remove the region containing the mismatch. DNA polymerase III fills in the gap, using the opposite strand as the template, and ligase joins the ends, to restore a continuous strand.

Eukaryotes also have a mismatch repair system that repairs not only single base mismatches but also insertions and deletions. Homologs to the E. coli MutS and MutL have been identified in other organisms, including humans: hMSH1 and hMSH2 (human MutS homolog 1 and 2) are homologous to MutS, while hMLH 1 is homologous to MutL. These, together with additional proteins, carry out mismatch repair in eukaryotic cells.

DNA methylation is not used by eukaryotic cells as a way to distinguish the new strand from the template, and it is not yet completely understood how the mismatch repair system in eukaryotes "knows" which strand to repair. There is evidence that the newly made DNA may be recognized by the fact that it is nicked, or discontinuous. This suggests that discontinuity resulting from Okazaki fragments that have not yet been joined together may permit the new strand to be distinguished from the old, continuous template strand.

Repairing damage to DNA

In the preceding section we looked at mistakes made when DNA is copied, where the wrong base is inserted during synthesis of the new strand. But even DNA that is not being replicated can get damaged or mutated. These sorts of damage are not associated with DNA replication, rather they can occur at any time.

What causes damage to DNA?
Some major causes of DNA damage are:
a. Radiation (e.g., UV rays in sunlight and in tanning booths, or ionizing radiation)
b. Exposure to damaging chemicals, such as nitrosamines or polycyclic aromatic hydrocarbons, in the environment (see Figure 7.38)
c. Chemical reactions within the cell (such as the deamination of cytosine to give uracil, or the methylation of guanine to produce methylguanine).

This means the DNA in your cells is vulnerable to damage simply from normal sorts of actions, such as walking outdoors, being in traffic, or from the chemical transformations occurring in every cell as part of its everyday activities. (Naturally, the damage is much worse in situations where exposure to radiation or damaging chemicals is greater, such as when people use tanning beds, or smoke, regularly.)

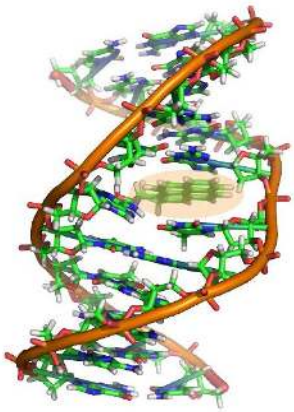


Figure 7.38 - DNA adducted to benz-o-pyrene (highlighted in orange)

Types of damage

What kinds of damage do these agents cause? Radiation can cause different kinds of damage to DNA.

Sometimes, as with much of the damage done by UV rays, two adjacent pyrimidine bases in the DNA will be cross-linked to form cyclobutane pyrimidine dimers or CPDs (see Figure 7.39). Note that these are two neighboring pyrimidine bases on the same strand of DNA. UV exposure can also lead to the formation of another type of lesion, known as a (6-4) photoproduct or 6-4PP (Figure 7.39). Ionizing radiation can cause breaks in the DNA backbone, in one or both strands.

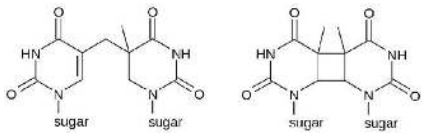


Figure 7.39 - Possible chemical structures of a pyrimidine dimer - 6-4PP (left) and CPD (right) - Wikipedia

Molecules like benzopyrene, found in automobile exhaust, can attach themselves to bases, forming bulky DNA adducts in which large chemical groups are linked to bases in the DNA. Damage like pyrimidine dimers, 6-4PPs or chemical adducts can physically distort the DNA helix, causing DNA and RNA polymerase to stall when they attempt to copy those regions of DNA (Figure 7.40).

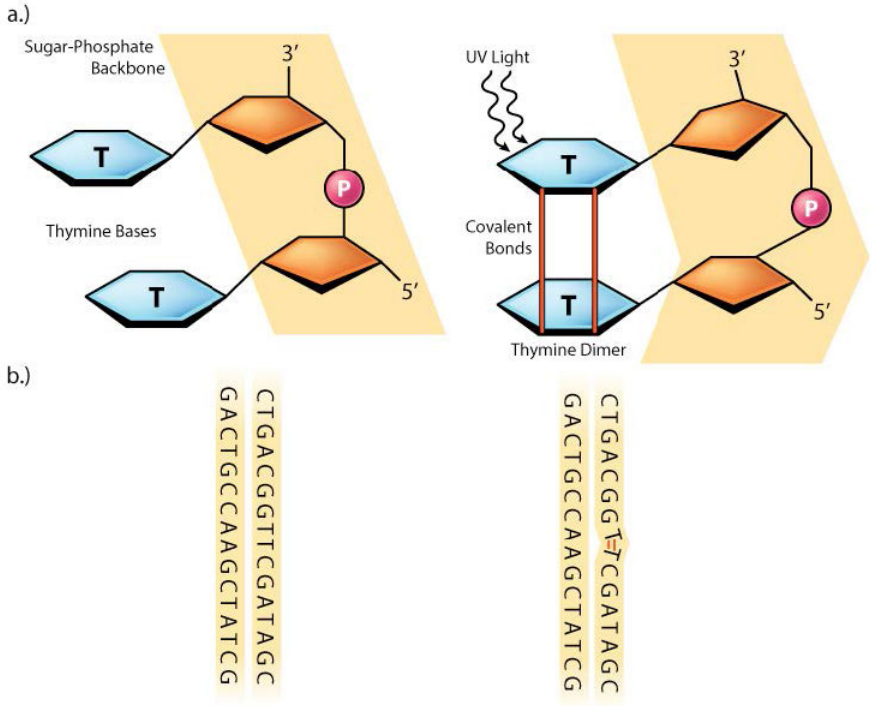


Figure 7.40- Thymine dimers distort the DNA helix -Aleia Kim

Chemical reactions occurring within cells can cause cytosines in DNA to be deaminated to uracil. Other sorts of damage in this category include the formation of oxidized bases like 8-oxo-guanine or alkylated bases like O6-methylguanine. These do not actually change the physical structure of the DNA helix, but they can cause problems because uracil and 8-oxo-guanine pair with different bases than the original cytosine or guanine, leading to mutations on the next round of replication. O6-methylguanine similarly can form base pairs with thymine instead of cytosine.

Removing damage

Cells have several ways to remove the sorts of damage described above. The first of these is described as **direct reversal**. Many organisms (though, unfortunately for us, not humans) can repair UV damage like CPDs and 6-4PPs because they possess enzymes called photolyases (photo=light; lyase=breakdown enzyme - Figure 7.41). Photolyases work through a process called photoreactivation, and use blue light energy to catalyze a photochemical reaction that breaks the aberrant bonds in the damaged DNA and returns the DNA to its original state.

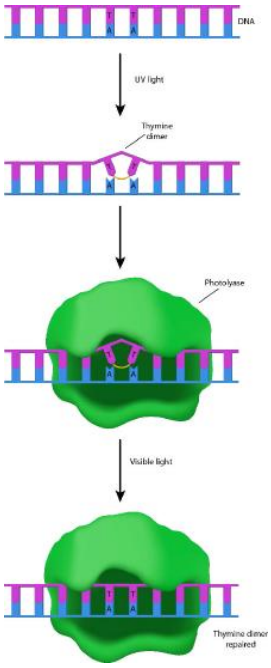


Figure 7.41- Direct repair of thymine dimers by photolyase - Pehr Jacobsen

Suicide enzyme

O6-methylguanine in DNA can also be removed by direct reversal, with the help of the enzyme O6-methylguanine methyltransferase. This is a very unusual enzyme that removes the methyl group from the guanine and transfers it onto a cysteine residue in the enzyme. The addition of the methyl group to the cysteine renders the enzyme non-functional.

As you know, most enzymes are catalysts that remain unchanged over the course of the reaction, permitting a single enzyme molecule to repeatedly catalyze a reaction. Because the O6-methylguanine methyltransferase does not fit this description, it is sometimes not regarded as a true enzyme. It has also been called a suicide enzyme, because the enzyme “dies” as a result of its own activity.

Excision repair

Excision repair is another common strategy. Excision repair is a general term for the cutting out and re-synthesizing of the damaged region of a DNA. There are several different kinds of excision repair, but they all involve excising the portion of the DNA that is damaged, followed by repair synthesis using the other strand as template, and finally, ligation to restore continuity to the repaired strand. Cells possess several different kinds of excision repair, each geared to specific kinds of DNA damage. Between them, these repair systems deal with the wide variety of insults to the genome.

Nucleotide excision repair

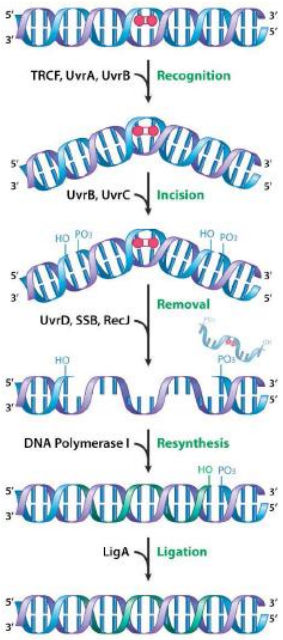


Figure 7.42 - Nucleotide excision repair - Aleia Kim

Nucleotide excision repair (NER) fixes damage such as the formation of chemical adducts, as well as UV damage. Both chemical adducts and the formation of CPDs or 6,4 photoproducts can cause significant distortion of the DNA helix. NER proteins act to cut the damaged strand on either side of the lesion. A short portion of the DNA strand containing the damage is then removed and a DNA polymerase fills in the gap with the appropriate nucleotides. Nucleotide excision repair has been extensively studied in bacteria.

In E. coli, recognition and excision of the damage is carried out by a group of proteins encoded by the uvrABC and uvrD genes. The protein products of the uvrA, uvrB and uvrC genes function together as the so-called UvrABC excinuclease. The damage is initially recognized and bound by a complex of the UvrA and UvrB proteins. Once the complex is bound, the UvrA dissociates, leaving the UvrB attached to the DNA, where it is then joined by the UvrC protein.

Strand nicking

It is the complex consisting of UvrB and C that acts to cut the phosphodiester backbone on either side of the damage, creating nicks in the strand about 12-13 nucleotides apart. A helicase encoded by uvrD then unwinds the region containing the damage, displacing it from the double helix together with UvrBC. The gap in the DNA is filled in by DNA polymerase, which copies the undamaged strand, and the nick is sealed with the help of DNA ligase.

Nucleotide excision repair is also an important pathway in eukaryotes. It is particularly important in the removal of UV damage in humans, given that we lack photolyases. A number of proteins have been identified that function in ways similar to the Uvr proteins.

The importance of these proteins is evident from the fact that mutations in the genes that encode them can lead to a number of genetic diseases, like Xeroderma pigmentosum, or XP. People with XP are extremely sensitive to UV exposure, because the damage caused by it cannot be repaired, leaving them at a much higher risk of developing skin cancer.

Two repair modes

Nucleotide excision repair operates in two modes, one known as global genomic repair and the other as transcription-coupled repair. While the function of both is to remove helix-destabilizing damage like cyclobutane pyrimidine dimers or chemical adducts, the way in which the lesions are detected differs.

In global genomic repair, damage is identified by surveillance of the entire genome for helix distorting lesions. In the case of transcription-coupled repair, the stalling of the RNA polymerase at a site of DNA damage is the indicator that activates this mode of nucleotide excision repair.

Base excision repair

Base excision repair (BER) is a repair mechanism that deals with situations like the deamination of cytosine to uracil (Figure 7.43) or the methylation of a purine base. These changes do not typically distort the structure of the DNA helix, unlike chemical adducts or UV damage.

In base excision repair a single damaged base is first removed from the DNA, followed by removal of a region of the DNA surrounding the missing base. The gap is then repaired.

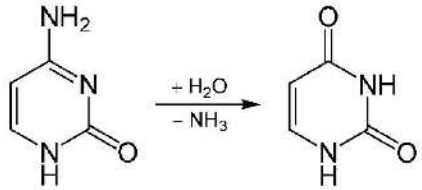


Figure 7.43 - Spontaneous hydrolysis of cytosine to form uracil

Uracil-DNA glycosylase

The removal of uracil from DNA is accomplished by the enzyme uracil-DNA glycosylase that can recognize uracil in DNA and break the glycosidic bond between the uracil and the sugar in the nucleotide (Figure 7.44). The removal of the base leaves a gap called an apyrimidinic site (AP site) because, in this case, uracil, a pyrimidine was removed. It is important to remember that at this point the backbone of the DNA is still intact, and the removal of a single base simply creates a gap like a tooth that has been knocked out.

The formation of the AP site triggers the activity of an enzyme known as an AP endonuclease that cuts the DNA backbone 5' to the AP site. In the remaining steps, a DNA polymerase binds to the nick, then using its exonuclease and polymerase activities, replaces the sequence in this region. Depending on the situation, a single nucleotide may be replaced (short patch BER) or a stretch of several nucleotides may be removed and replaced (long patch BER). Finally, as always, DNA ligase acts to seal the nick in the DNA.

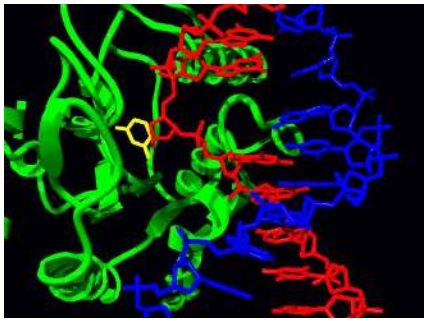


Figure 7.44 - Base excision repair by uracil-DNA glycosylase. The yellow uracil base has been "flipped" out of the DNA helix for excision

Repair of double-strand breaks

While all the repair mechanisms discussed so far fixed damage on one strand of DNA using the other, undamaged strand as a template, these mechanisms cannot repair damage to both strands. What happens if both strands are damaged? Ionizing radiation, exposure to certain chemicals, or reactive oxygen species generated in the cell can lead to double-strand breaks (DSBs) in DNA.

DSBs are a potentially lethal form of damage that, in addition to blocking replication and transcription, can also lead to chromosomal translocations, where part of one chromosome gets attached to a piece of another chromosome. Two different cellular mechanisms exist that help repair DSBs (Figure 7.45), homologous recombination (HR) and non-homologous end joining (NHEJ).

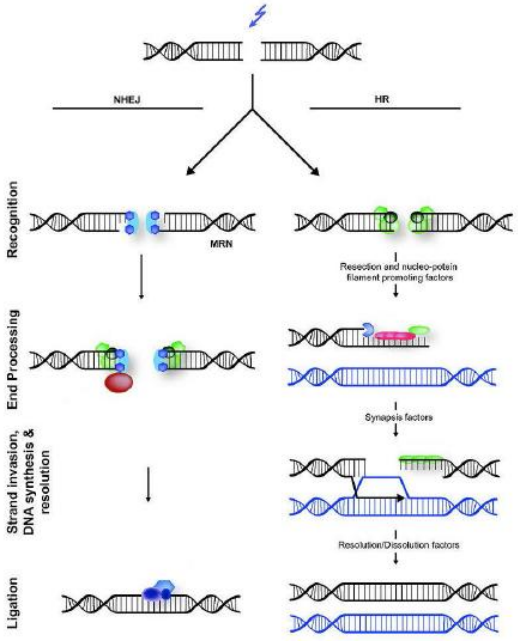


Figure 7.45 - Non-homologous end joining (left) versus homologous recombination (right) - Wikipedia

Homologous recombination repair commonly occurs in the late S and G2 phases of the cell, when each chromosome has been replicated and information from a sister chromatid can be used as a template to achieve error-free repair. Note that in contrast to excision repair, where the damaged strand was removed and the undamaged sister strand served as the template for filling in the damaged region, HR must use the information from another DNA molecule, because both strands of the DNA are damaged in DSBs.

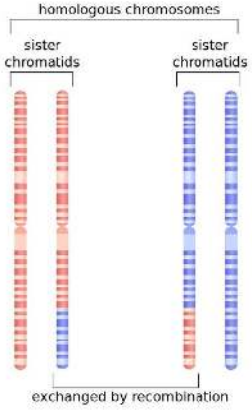


Figure 7.46 - Result of homologous recombination - Wikipedia

Nuclease action

Detection of the double-strand break triggers nuclease activity that chews back one strand on each end of the break. This results in the production of single-stranded 3’ overhangs on each end. These single-stranded ends are bound by several proteins, creating a nucleoprotein filament that can then “search” for homologous (matching) sequences on a sister chromatid.

When such sequences are found, the nucleoprotein filament invades the undamaged sister chromatid, forming a crossover. This creates heteroduplexes made up of DNA strands from different chromatids. Strand invasion (Figure 7.47) is followed by branch migration, during which the Holliday junction moves along the DNA, extending the heteroduplex away from the original site of the crossover (Figure 7.48). In E. coli, branch migration depends on the activity of two proteins, RuvA and RuvB. The resulting recombination intermediate can be resolved, with the help of RuvC to give complete, error-free strands.

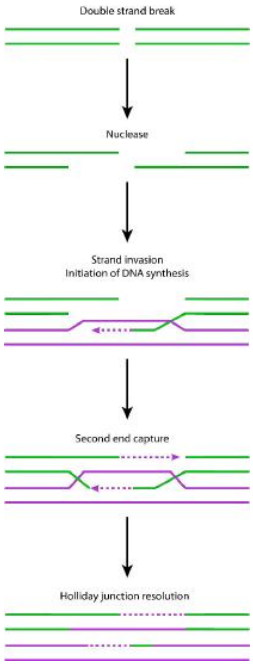


Figure 7.47 - Repair of DSBs by homologous recombination - Image by Pehr Jacobson

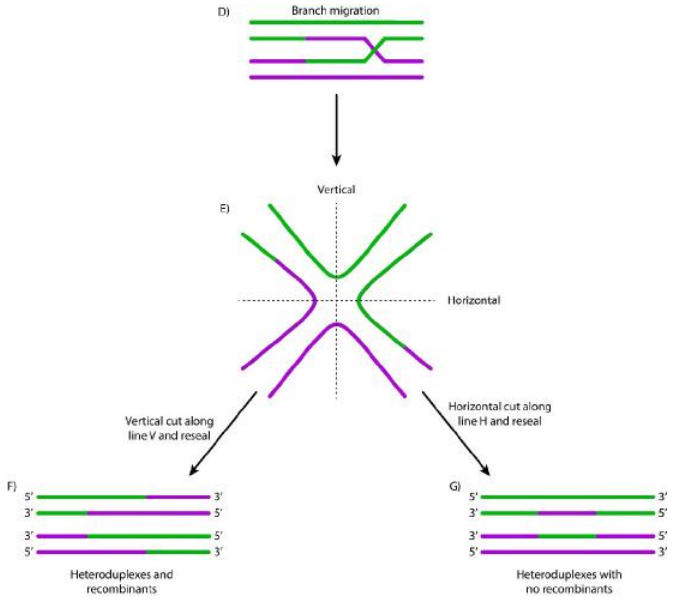


Figure 7.48 - Resolution of Holliday Junctions - Image by Pehr Jacobsen

Non-homologous end joining

In contrast to homologous recombination, Non-homologous end joining (NHEJ) is error-prone. It does not use or require a homologous template to copy, and works by simply chewing back the broken ends of DSBs and ligating them together. Not surprisingly, NHEJ introduces deletions in the DNA as a result.

Translesion DNA synthesis

As we have seen, cells have a variety of mechanisms to help safeguard the integrity of the information in DNA. One measure of last resort is translesion DNA synthesis, also known as bypass synthesis. Translesion synthesis occurs when a DNA polymerase encounters DNA damage on the template strand, but instead of stalling or skipping past the damage, replication switches to an error-prone mode, ignoring the template and incorporating random nucleotides into the new strand. In E.coli, translesion synthesis is dependent on the activities of proteins encoded by the umuC and umuD genes. Under the appropriate conditions (see SOS

response, below) UmuC and UmuD are activated to begin bypass synthesis. Being error-prone, translesion synthesis gives rise to many mutations.

The SOS response

Named for standard SOS distress signals, the term “SOS repair” refers to a cellular response to UV damage. When bacterial cells suffer extensive damage to their DNA as a result of UV exposure, they turn on the coordinated expression of a large number of genes that are necessary for DNA repair. These include the uvr genes needed for nucleotide excision repair and recA, which is involved in homologous recombination. In addition to these mechanisms, which can carry out error-free repair, the SOS response can also induce the expression of translesion polymerases encoded by the dinA, dinB and umuCD genes.

How are all these genes induced in a coordinated way following UV damage? All of the genes induced in the SOS response are regulated by two components. The first is the presence of a short DNA sequence upstream of their coding region, called the SOS box. The second is a protein, the LexA repressor (Figure 7.49), that binds to the SOS box and prevents transcription of the downstream genes. Expression of the genes requires the removal of LexA from its binding site. How is this achieved?

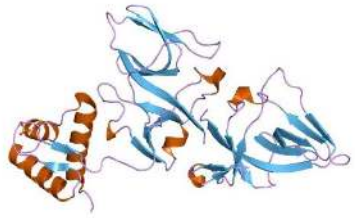


Figure 7.49 -Lex A repressor, N-terminal domain

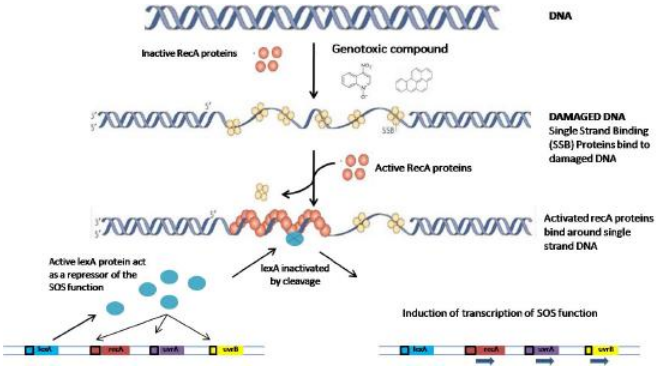


Figure 7.50 - Activation of SOS genes by lexA in E. coli

When exposure to radiation results in DNA breaks, the presence of single-stranded regions triggers the activation and binding of RecA proteins to the single-stranded region, creating a nucleoprotein filament. The interaction of the RecA with the LexA repressor leads to autocleavage of the repressor, allowing the downstream gene(s) to be expressed (Figure 7.50).

The genes controlled by the LexA repressor, as mentioned earlier, encode proteins that are necessary for accurate DNA repair as well as error-prone translesion synthesis. The various genes involved in DNA repair are induced in a specific order. In the initial stages, the repair genes that are derepressed are for nucleotide excision repair, followed by homologous recombination, both error-free mechanisms for repair. If the damage is too extensive to be repaired by these systems, error-prone repair mechanisms may be brought into play as a last resort.

SOS response and antibiotic resistance

The increased mutation rate in the SOS response may play a role in the acquisition of antibiotic resistance in bacteria (Figure 7.51).

An example is the development of resistance to topoisomerase poisons like the fluoroquinolone family of drugs. Fluoroquinolones inhibit the ability of topoisomerases to religate the ends of their substrates after nicking them to allow overwound DNA to relax. This results in accumulation of strand breaks that can trigger the SOS response. As a consequence of error-prone DNA synthesis by

low fidelity polymerases during the SOS response, there is a large increase in the number of mutations. While some mutations may be lethal to the bacteria, others can contribute to the rapid development of drug resistance in the population.

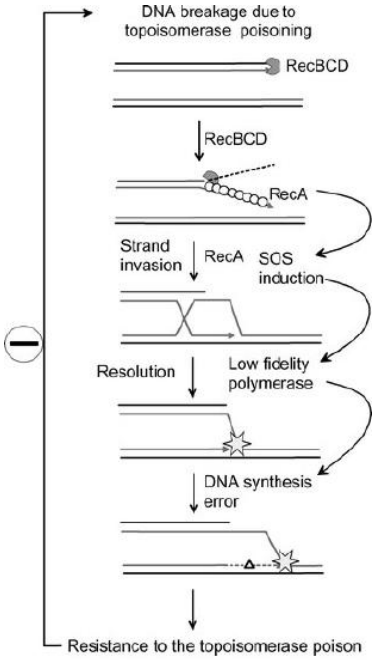


Figure 7.51 - A proposed mechanism of evolved bacterial antibiotic resistance - Wikipedia

---

This page titled 4.4: DNA Repair is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **7.4: DNA Repair** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

4.5: Genes and Genomes

Source: BiochemFFA_7_1.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy



Introduction

For many years, scientists wondered about the nature of the information that directed the activities of cells. What kind of molecules carried the information, and how was the information passed on from one generation to the next? Key experiments, done between the 1920s and the 1950s, established convincingly that this genetic information was carried by DNA. In 1953, with the elucidation of the structure of DNA, it was possible to begin investigating how this information is passed on, and how it is used.

Genomes

We use the word “genome” to describe all of the genetic material of the cell. That is, a genome is the entire sequence of nucleotides in the DNA that is in all of the chromosomes of a cell. When we use the term genome without further qualification, we are generally referring to the chromosomes in the nucleus of a eukaryotic cell. As you know, eukaryotic cells have organelles like mitochondria and chloroplasts that have their own DNA (Figure 7.1 & 7.2). These are referred to as the mitochondrial or chloroplast genomes to distinguish them from the nuclear genome.

Starting in the 1980s, scientists began to determine the complete sequence of the genomes of many organisms, in the hope of better understanding how the DNA sequence specifies cellular functions. Today, the complete genome sequences have been determined for thousands of species from all domains of life, and many more are in the process of being worked out by groups of scientists across the world.

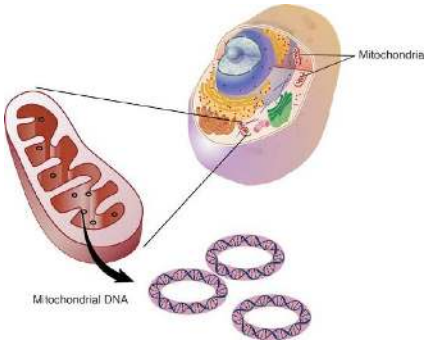


Figure 7.1 - Mitochondria carry their own genome

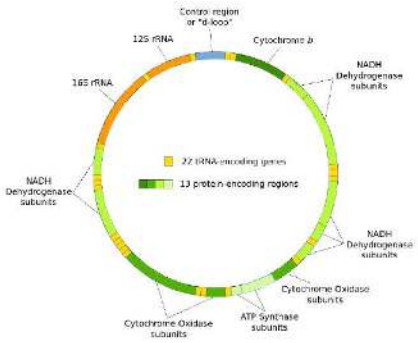


Figure 7.2 - The human mitochondrial genome - Wikipedia

Global genome initiative

The Global Genome Initiative, a collaborative effort to sequence at least one species from each of the 9,500 described invertebrate, vertebrate, and plant families is one of many such ventures. The information from these various efforts is collected in enormous online repositories, so that it is freely available to scientists. As the sequence databases compile ever more information, the fields of computational biology and bioinformatics have arisen, to analyze and organize the data in a way that helps biologists understand what the information in DNA means in the cellular context.

Genes

It has been known for many years that phenotypic traits are controlled by specific regions of the DNA that were termed “genes”. Thus, DNA was envisioned as a long string of nucleotides, in which certain regions, the genes, were separated by non-coding regions that were simply referred to as intergenic sequences (inter=between; genic=of genes). Early experiments in molecular biology suggested a simple relationship between the DNA sequence of a gene and its product, and led scientists to believe that each gene carried the information for a single protein. Changes, or mutations in the base sequence of a gene would be reflected in changes in the gene product, which in turn, would manifest itself in the phenotype or observable trait. This simple picture, while still useful, has been modified by subsequent discoveries that demonstrated that the use of genetic information by cells is somewhat more complicated. Our definition of a gene is also evolving to take new knowledge into consideration.

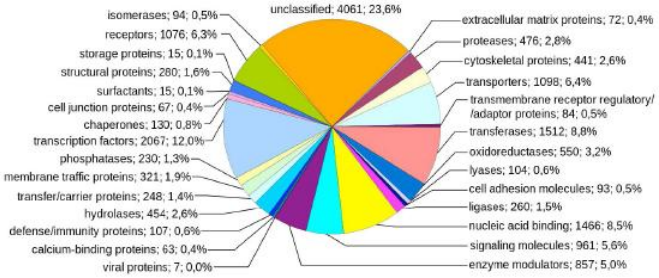


Figure 7.3 - From genomes to genes - Wikipedia

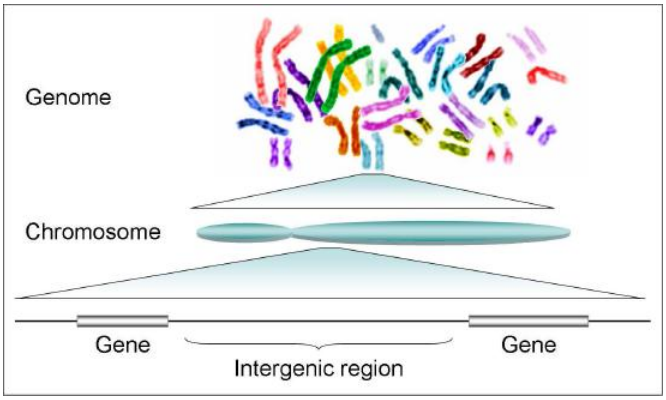


Figure 7.4 - Human genes sorted by class

Matters of size

A common-sense assumption about genomes would be that if genes specify proteins, then the more proteins an organism made, the more genes it would need to have, and thus, the larger its genome would be. Comparison of various genomes shows, surprisingly, that there is not necessarily a direct relationship between the complexity of an organism and the size of its genome (Figure 7.5). To understand how this could be true, it is necessary to recognize that while genes are made up of DNA, all DNA does not consist of genes (for purposes of our discussion, we define a gene as a section of DNA that encodes an RNA or protein product). In the human genome, less than 2% of the total DNA seems to be the sort of coding sequence that directs the synthesis of proteins. For many years, non-coding DNA in genomes was believed to be useless, and was described as "junk DNA" although it was perplexing that there seemed to be so much "useless" sequence. Recent discoveries have, however, demonstrated that much of this so-called junk DNA may play important roles in evolution, as well as in regulation of gene expression.

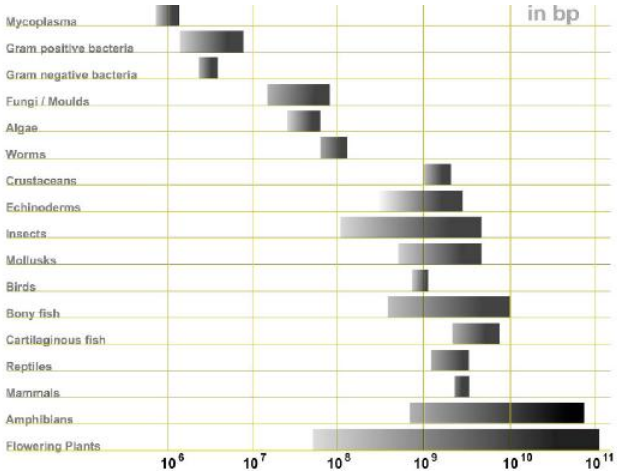


Figure 7.5 - Sizes of various genomes - Wikipedia

Introns

So, what is all the non-coding DNA doing there? We know that even coding regions in our DNA are interrupted by non-coding sequences called introns. This is true of most eukaryotic genomes. An examination of genes in eukaryotes shows that non-coding intron sequences can be much longer than the coding sections of the gene, or exons. Most exons are relatively small, and code for fewer than a hundred amino acids, while introns can vary in size from several hundred base-pairs to many kilobase-pairs (thousands of base-pairs) in length. For many genes in humans, there is much more of intron sequence than coding (a.k.a. exon) sequence. Intron sequences account for roughly a quarter of the genome in humans.

Other non-coding sequences

What other kinds of non-coding sequences are there? One function for some DNA sequences that do not encode RNA or proteins is in specifying when and to what extent a gene is used, or expressed. Such regions of DNA are called regulatory regions and each gene has one or more regulatory sequences that control its expression. However, regulatory sequences do not account for all the rest of the DNA in our genomes, either.

Transposable sequences

Surprisingly, almost half of the human genome appears to consist of several kinds of repetitive sequences. Many of the repetitive sequences are known to be transposable elements (transposons), sections of DNA that can move around within the genome. Sometimes referred to as "jumping genes" these transposable elements can move from one chromosomal location to another, either through a simple "cut and paste" mechanism that cuts the sequence out of one region of the DNA and inserts it into another location, or through a process called retrotransposition involving an RNA intermediate.

LINES & SINES

There are millions of copies of each of two major classes of such transposable elements, the LINEs (Long Interspersed Elements) and SINEs (Short Interspersed Elements) in our genomes.

LINEs and SINEs are both a kind of transposable element called retrotransposons, sequences that are copied into RNA, then reverse transcribed back into DNA before being inserted into new locations. This movement is typically not sequence specific, meaning that the transposons can be inserted randomly in the genome, in many cases within coding regions. As might be expected, this can disrupt the function of the gene. Transposons may also insert within regulatory regions, and change the expression of the genes they control. As a major cause of mutation in genomes, transposons play an important role in evolution.

Finally, recent findings have shown that much of the genome is transcribed into RNAs, even though only about 2% encodes proteins. What are the RNAs that do not encode proteins? Ribosomal RNAs (Figure 7.7) and transfer RNAs, together with the small nuclear RNAs that function in splicing, account for some of these non-translated transcripts, but not all. The remaining RNAs are regulatory RNAs, small molecules that play an important role in regulating gene expression. As we understand more about genomes, it is becoming evident that the so-called “junk” DNA is anything but.

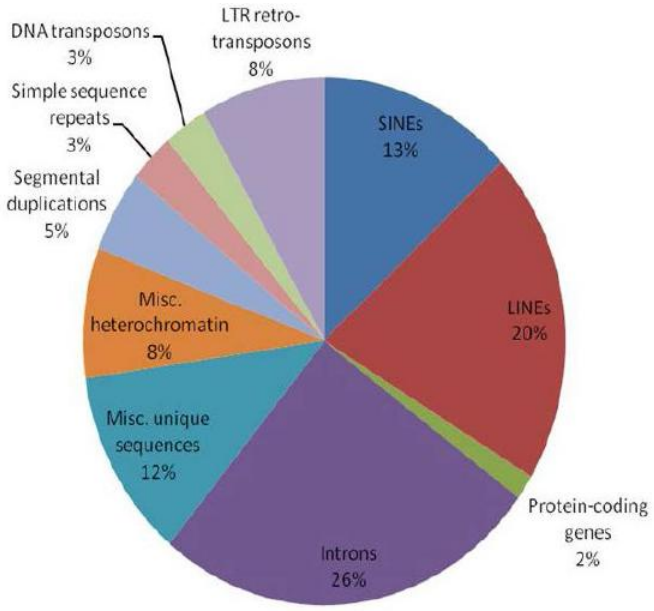


Figure 7.6 - Components of the human genome - Wikipedia

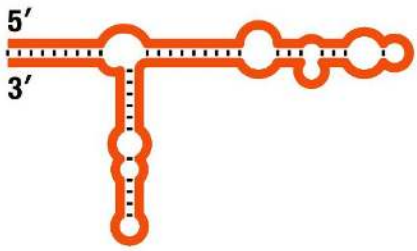


Figure 7.7 - 5S rRNA structure

This page titled 4.5: Genes and Genomes is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **7.2: Genes and Genomes** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

4.6: Transcription

Source: BiochemFFA_7_4.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

In the preceding sections, we have discussed the replication of the cell's DNA and the mechanisms by which the integrity of the genetic information is carefully maintained. What do cells do with this information? How does the sequence in DNA control what happens in a cell? If DNA is a giant instruction book containing all of the cell's "knowledge" that is copied and passed down from generation to generation, what are the instructions for? And how do cells use these instructions to make what they need?

Information flow

You have learned in introductory biology courses that genes, which are instructions for making proteins, are made of DNA. You also know that information in genes is copied into temporary instructions called messenger RNAs that direct the synthesis of specific proteins. This description of flow of information from DNA to RNA to protein is often called the central dogma of molecular biology and is a good starting point for an examination of how cells use the information in DNA.

Consider that all of the cells in a multicellular organism have arisen by division from a single fertilized egg and therefore, all have the same DNA. Division of that original fertilized egg produces, in the case of humans, over a trillion cells, by the time a baby is produced from that egg (that's a lot of DNA replication!). Yet, we also know that a baby is not a giant ball of a trillion identical cells, but has the many different kinds of cells that make up tissues like skin and muscle and bone and nerves. How did cells that have identical DNA turn out so different?

The answer lies in gene expression, which is the process by which the information in DNA is used. Although all the cells in a baby have the same DNA, each different cell type uses a different subset of the genes in that DNA to direct the synthesis of a distinctive set of RNAs and proteins. The first step in gene expression is transcription, which we will examine next (Figure 7.52).

Transcription

Transcription is the process of copying information from DNA sequences into RNA sequences. This process is also known as DNA-dependent RNA synthesis. When a sequence of DNA is transcribed, only one of the two DNA strands is copied into RNA. We will consider what determines which strand of DNA is copied into RNA, later on.

But, apart from copying one, rather than both strands of DNA, how is transcription different from replication of DNA? DNA replication serves to copy all the genetic material of the cell and occurs before a cell divides, so that a full copy of the cell's genetic information can be passed on to the daughter cell. Transcription, by contrast, copies short stretches of the coding regions of DNA to make RNA. Different genes may be copied into RNA at different times in the cell's life cycle. RNAs are, essentially, temporary copies of the information in DNA and different sets of instructions are copied for use at different times and in different cell types.

Cells make several different kinds of RNA:

- mRNAs that code for proteins
- rRNAS that form part of ribosomes
- tRNAs that serve as adaptors between mRNA and amino acids during translation
- Small RNAs that regulate gene expression, including miRNAs and siRNAs
- Other small RNAs that have a variety of functions, including the small nuclear RNAs that are part of the splicing machinery.
- Long noncoding RNAs (lnc RNAs - Figure 7.53)

Building an RNA strand is very similar to building a DNA strand. This is not surprising, knowing that DNA and RNA are very similar molecules. Transcription is catalyzed by the enzyme RNA Polymerase. "RNA polymerase" is a general term for an enzyme that makes RNA. There are several different kinds of RNA polymerases in eukaryotic cells, while in prokaryotes, a single type of RNA polymerase is responsible for all transcription.

RNA synthesis

Like DNA polymerases, RNA polymerases synthesize new strands only in the 5' to 3' direction, but because they are making RNA, they use ribonucleotides (i.e., RNA nucleotides - Figure 7.54) rather than deoxyribonucleotides. Ribonucleotides are joined in

exactly the same way as deoxyribonucleotides, i.e., the 3'OH of the last nucleotide on the growing chain is joined to the 5' phosphate on the incoming nucleotide to make a phosphodiester bond.

One important difference between DNA polymerases and RNA polymerases is that the latter do not require a primer to start making RNA. Once RNA polymerases are in the right place to start copying DNA, they just begin making RNA by joining together RNA nucleotides complementary to the DNA template.

Starting points

This, of course, brings us to an obvious question- how do RNA polymerases "know" where to start copying on the DNA?

Unlike the situation in replication, where every nucleotide of the parental DNA must eventually be copied, transcription, as we have already noted, only copies selected portions of the DNA into RNA at any given time. Consider the challenge here: in a human cell, there are approximately 6 billion base-pairs of DNA. Much of this is non-coding DNA, meaning that it will not need to be transcribed. The small percentage of the genome that is made up of coding sequences still amounts to between 20,000 and 30,000 genes in each cell. Of these genes, only a small number will need to be expressed at any given time.What indicates to an RNA polymerase where to start copying DNA to make a transcript?

Promoters

It turns out that patterns in the DNA sequence indicate where RNA polymerase should start and end transcription. These sequences are recognized by the RNA polymerase or by proteins that help RNA polymerase determine where it should bind the DNA to start transcription. A DNA sequence at which the RNA polymerase binds to start transcription is called a promoter. The DNA sequence that indicates the endpoint of transcription, where the RNA polymerase should stop adding nucleotides and dissociate from the template is known as a terminator sequence. The promoter and terminator, thus, bracket the region of the DNA that is to be transcribed.

A promoter is described as being situated upstream of the gene that it controls (Figure 7.57). What this means is that on the DNA strand that the gene is on, the promoter sequence is "before" the gene, or to put it differently, it is on the side of the gene opposite to the direction of transcription. Also notice that the promoter is said to "control" the gene it is associated with. This is because expression of the gene is dependent on the binding of RNA polymerase to the promoter sequence to begin transcription. If the RNA polymerase and its helper proteins do not bind at the promoter, the gene cannot be transcribed and it will therefore, not be expressed.

What is special about a promoter sequence? In an effort to answer this question, scientists examined many genes and their surrounding sequences (Figure 7.57). Because the same RNA polymerase has to bind to many different promoters, it would be predicted that promoters would have some similarities in their sequences. As expected, common sequence patterns were seen to be present in many promoters.

We will first take a look at prokaryotic promoters.

When prokaryotic genes were examined, the following features commonly emerged:

- A transcription start site (this the base in the DNA across from which the first RNA nucleotide is paired), which, by convention, is denoted as +1.
- A -10 sequence: this is a 6 bp region centered about 10 bp upstream of the start site. The consensus sequence at this position is TATAAT. In other words, if you count back from the transcription start site, the sequence found at roughly -10 in the majority of promoters studied is TATAAT.
- A -35 sequence: this is a 6 bp sequence at about 35 basepairs upstream from the start of transcription. The consensus sequence at this position is TTGACA.

It is important to understand that each nucleotide in a consensus sequence is simply the one that appeared at that position in the majority of promoters examined, and does not mean that the entire consensus sequence is found in all promoters. In fact, few promoters have -10 and -35 sequences that exactly match the consensus. The box at the left shows the -10 and -35 sequences by percentage of occurrence of each base in the promoter.

What is the significance of these sequences? It turns out that the sequences at -10 and -35 are necessary for recognition of the promoter region by RNA polymerase (Figure 7.58). The sequences at -10 and -35 may vary a little in individual promoters, as

mentioned above, but the extent to which they are different is limited. It is only when the RNA polymerase has stably bound at the promoter that transcription can begin. The process by which the promoter is recognized and bound stably has been well studied for the RNA polymerase of E. coli.

Core polymerase and holoenzyme

The E. coli RNA polymerase is made up of a core enzyme of five subunits (α2ββ'and ω) and an additional subunit called the σ (sigma) subunit. Together, the σ subunit and core polymerase make up what is termed the RNA polymerase holoenzyme. The core polymerase is the part of the RNA polymerase that is responsible for the actual synthesis of the RNA, while the σ subunit is necessary for binding of the enzyme at promoters to initiate transcription.

Loose association

The core polymerase and σ subunit are not always associated with each other. For the most part, the core polymerase is loosely associated with DNA, although it does not discriminate between promoters and other sequences in DNA, and the DNA strands are not opened up to allow transcription in this state. The role of the σ subunit is to reduce the affinity of the core polymerase for non-specific DNA sequences and to help the enzyme specifically bind to promoter sequences.

Holoenzyme binding

It is when the σ subunit associates with the core polymerase that the holoenzyme is able to bind specifically to promoter sequences. The initial binding of the holoenzyme at the promoter results in what is called a "closed" complex, meaning that the DNA template is still double-stranded and has not opened up to allow transcription. This closed complex is then converted to an "open" complex by the separation of the DNA strands to create a transcription bubble about 12-14 base-pairs long (Figure 7.60). The conversion of the closed complex to the open complex also requires the presence of the σ subunit.

Open complex & initiation

Once the open complex has formed, the DNA template can begin to be copied, and the core polymerase adds nucleotides complementary to one strand of the DNA. At this stage, known as initiation, the polymerase adds several nucleotides while still bound to the promoter, and without moving along the DNA template. Initially, short pieces of RNA a few nucleotides long may be made and released, without the polymerase leaving the promoter. Eventually, the enzyme makes the transition to the next stage, elongation, when an RNA of 8-9 bases is made and the enzyme moves beyond the promoter region.

Elongation

Once elongation commences and the RNA polymerase is moving down the DNA template, the σ subunit is no longer necessary and may dissociate from the core enzyme. The core polymerase can move along the template, unwinding the DNA ahead of it to maintain a transcription bubble of 12-15 base-pairs and synthesizing RNA complementary to one of the strands of the DNA. As already mentioned, an RNA chain, complementary to the DNA template, is built by the RNA polymerase by the joining of the 5' phosphate of an incoming ribonucleotide to the 3'OH on the last nucleotide of the growing RNA strand. Behind the RNA polymerase, the DNA template is rewound, displacing the newly made RNA from its template strand.

Termination

As mentioned earlier, a sequence of nucleotides called the terminator is the signal to the RNA polymerase to stop transcription and dissociate from the template. Some terminator sequences, known as intrinsic terminators, allow termination by RNA polymerase without the help of any additional factors, while others, called rho-dependent terminators, require the assistance of a protein factor called rho (ρ).

How does the sequence of the terminator cause the RNA polymerase to stop adding nucleotides and release the transcript?

To understand this, it is useful to know that the terminator sequence precedes the last nucleotide of the transcript. In other words, the terminator is part of the end of the sequence that is transcribed (Figure 7.61).

Intrinsic terminators

In intrinsic terminators, this sequence in the RNA has self-complementary regions that can base-pair with each other to form a hairpin structure that contains a GC-rich run in the "stem" of the hairpin. This hairpin is followed by a single-stranded region that is rich in U's (Figure 7.62). The secondary structure formed by the folding of the end of the RNA into the hairpin causes the RNA

polymerase to pause. Meanwhile, the run of U's at the end of the hairpin permits the RNA-DNA hybrid in this region to come apart, because the base-pairing between A's in the DNA template and the U's in the RNA is relatively weak. This allows the transcript to be released from the DNA template and from the RNA polymerase.

Rho-dependent termination

In the case of rho-dependent termination, an additional protein factor, rho, is necessary. Rho is a helicase that can separate the transcript from the template it is paired with. As in intrinsic termination, rho-dependent termination requires the formation of a hairpin structure in the RNA that causes pausing of the RNA polymerase. Meanwhile, rho binds to a region of the transcript called the rho utilization site (rut) and moves along the RNA till it reaches the paused RNA polymerase. It then acts on the RNA-DNA hybrid, releasing the transcript from the template.

Coupled transcription and translation

In prokaryotes, which lack a nucleus, the DNA is not separated from the rest of the cell in a separate compartment, so the mRNA is immediately available to the translation machinery, as the transcript is coming off the template DNA. Indeed, in prokaryotic cells, translation of the mRNA can begin before the entire gene has been transcribed. Ribosomes can assemble at the 5' end of the transcript, as it is displaced from the template, while the 3' end of the gene is still being copied. The lag time between transcription and translation is thus, very short in prokaryotes.

Transcription in eukaryotes

Although the process of RNA synthesis is the same in eukaryotes as in prokaryotes, there are some additional considerations in eukaryotes. One is that in eukaryotes, the DNA template exists as chromatin, where the DNA is tightly associated with histones and other proteins. The "packaging" of the DNA must therefore be opened up to allow the RNA polymerase access to the template in the region to be transcribed (Figure 7.63). The restructuring of chromatin to allow access to regions of DNA is thus an important factor in determining which genes are expressed.

Multiple RNA polymerases

A second difference is that eukaryotes have multiple RNA polymerases, not just one as in bacterial cells. The different eukaryotic polymerases transcribe different classes of genes. For example, RNA polymerase I transcribes the ribosomal RNA genes, while RNA polymerase III copies tRNA genes. The RNA polymerase we will focus on most is RNA polymerase II, which transcribes protein-coding genes to make mRNAs.

All three eukaryotic RNA polymerases need additional proteins to help them get transcription started. In prokaryotes, RNA polymerase by itself can initiate transcription (the σ subunit is a subunit of the RNA polymerase, not an entirely separate protein). The additional proteins needed by eukaryotic RNA polymerases are referred to as transcription factors. We will see below that there are various categories of transcription factors.

Transcription and translation are de-coupled

Finally, in eukaryotic cells, transcription is separated in space and time from translation. Transcription happens in the nucleus, and the RNAs produced are processed further before they are sent into the cytoplasm.
Protein synthesis (translation) happens in the cytoplasm. As noted earlier, in prokaryotic cells, mRNAs can be translated as they are coming off the DNA template, and because there is no nuclear envelope, transcription and protein synthesis occur in a single cellular compartment. A representative eukaryotic gene, depicted in Figure 7.64 shows that transcription starts some 25 bp downstream of the TATA box, and creates a transcript that begins with a 5' untranslated region (5'UTR) followed by the coding region which may include multiple introns and ending in a 3' untranslated region or 3'UTR (Figure 7.64). As detailed below, the initial transcript is further processed before it is used.

Eukaryotic promoters

Like genes in prokaryotes, eukaryotic genes also have promoters that determine where transcription will begin. As with prokaryotes, there are specific sequences in the promoter regions that are recognized and bound by proteins involved in the initiation of transcription. We will focus primarily on the genes encoding proteins that are transcribed by RNA polymerase II. Such promoters commonly have a TATA box, a sequence similar to the -10 sequence in prokaryotic promoters. The TATA box is a sequence about 25-35 basepairs upstream of the start of transcription (+1). (Some eukaryotic promoters lack TATA boxes, and

have, instead, other recognition sequences, known as DPE, or downstream promoter elements.) Interestingly, the TATA box is not directly recognized and bound by RNA polymerase II. Instead, this sequence is bound by other proteins that, together with the RNA polymerase, form the transcription initiation complex.

Eukaryotic promoters also have, in addition, several other short stretches of sequences, that affect transcription, within about 100 to 200 base-pairs upstream of the transcription start site. These sequences, which are sometimes called upstream elements or promoter-proximal upstream elements, are bound by activator proteins that interact with the transcription complex that forms at the TATA box. Examples of such upstream elements are the CAAT box and the GC box (Figure 7.64).

Making transcripts in eukaryotes

We noted earlier that all eukaryotic RNA polymerases need additional proteins to bind promoters and start transcription. The proteins that help eukaryotic RNA polymerases find promoter sites and initiate RNA synthesis are termed general transcription factors. We will focus on the transcription factors that assist RNA polymerase II, the enzyme that transcribes protein-coding genes. These transcription factors are named TFIIA, TFIIB and so on (TF= transcription factor, II=RNA polymerase II, and the letters distinguish individual transcription factors).

Transcription by RNA polymerase II requires the general transcription factors and the RNA polymerase to form a complex, at the TATA box, called the basal transcription complex or transcription initiation complex (Figure 7.65).

This is the minimum requirement for any gene to be transcribed. The first step in the formation of this complex is the binding of the TATA box by a transcription factor, TFIID. TFIID is made up of several proteins, one of which is called the TATA Binding Protein or TBP. Binding of the TBP causes the DNA to bend at this spot and take on a structure that is suitable for the binding of additional transcription factors and RNA polymerase.

Interestingly, the binding of the TBP is a necessary step in forming a transcription initiation complex even when the promoter lacks a TATA box. The order of binding of additional proteins after binding of the TBP, as determined through in vitro experiments, appears to be TFIIB, followed by TFIIF and RNA polymerase II, then TFIIE. The final step in the assembly of the basal transcription complex is the binding of a general transcription factor called TFIIH. Some evidence suggests that following the binding of the TBP to DNA, the rest of the proteins in the initiation complex may assemble as a very large complex that then binds directly to the DNA. In any case, the presence of all of these general transcription factors and RNA Polymerase II bound at the promoter is necessary for the initiation of transcription.

As in prokaryotic transcription, once the RNA polymerase binds, it can begin to assemble a short stretch of RNA. This must be followed by promoter clearance, in order to move down the template and elongate the transcript. This requires the action of TFIIH. TFIIH is a multifunctional protein that has helicase activity (i.e., it is capable of opening up a DNA double helix) as well as kinase activity. The kinase activity of TFIIH adds phosphates onto the C-terminal domain (CTD) of the RNA polymerase II. This phosphorylation appears to be the signal that releases the RNA polymerase from the basal transcription complex and allows it to move forward on the template, building the new RNA as it goes (Figure 7.66).

Termination of transcription is not as well understood as it is in prokaryotes. Termination does not occur at a fixed distance from the 3’ end of mature RNAs. Rather, it seems to occur hand in hand with the processing of the 3’ end of the primary transcript. The polyadenylation signal in the 3’ untranslated region of the transcript appears to play a role in RNA polymerase pausing, and subsequent release of the completed primary transcript. Recognition of the polyadenylation signal triggers the binding of proteins involved in 3’end processing and termination.

Information Processing: Transcription

748

YouTube Lectures

by Kevin

HERE & HERE

749

Figure 7.52 - Overview of eukaryotic transcription

Wikipedia

750

Figure 7.53 - Transcripts may code for protein or may be functional as RNAs

Wikipedia

751

Figure 7.55 - RNA (green) being synthesized from DNA template (blue strand) by T7 RNA polymerase (purple). The non-template DNA strand is in red.

Figure 7.54 - The four ribonucleotides for making RNA

752

Figure 7.56 - Central dogma - DNA to RNA to protein

Wikipedia

753

YouTube Lectures

by Kevin

HERE & HERE

Figure 7.57 - Sequences upstream of transcription start site in several prokaryotic genes

Image by Martha Baker

754

-10 Sequence

T A T A A T

77% 76% 60% 61% 56% 82%

-35 Sequence

T T G A C A

69% 70% 61% 56% 54% 54%

Figure 7.58 - RNA polymerase promoter binding

Wikipedia

Figure 7.59 - A bacterial RNA polymerase (α2ββ’and ω)

755

Figure 7.60 - Synthesis of RNA in the transcription bubble

756

Figure 7.61 - Promoter and Terminator sequences determine where transcription starts and ends.

757

Figure 7.62 Transcription termination by intrinsic (top) and rho-dependent (bottom) mechanisms

Wikipedia

758

Figure 7.63 - Eukaryotic DNA is complexed with proteins in chromatin

Wikipedia

YouTube Lectures

by Kevin

HERE & HERE

759

Figure 7.64 - Region surrounding the transcriptional start site in eukaryotic DNA

Figure 7.65 - Transcription pre-initiation complex in eukaryotes

Wikipedia

760

761

This page titled 4.6: Transcription is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **7.5: Transcription** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

4.6.1: RNA Processing

Source: BiochemFFA_7_5.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

So far, we have looked at the mechanism by which the information in genes (DNA) is transcribed into RNA. The newly made RNA, also known as the primary transcript is further processed before it is functional. Both prokaryotes and eukaryotes process their ribosomal and transfer RNAs. The major difference in RNA processing, however, between prokaryotes and eukaryotes, is in the processing of messenger RNAs. We will focus on the processing of mRNAs in this section. You will recall that in bacterial cells, the mRNA is translated directly as it comes off the DNA template. In eukaryotic cells, RNA synthesis, which occurs in the nucleus, is separated from the protein synthesis machinery, which is in the cytoplasm. The initial product of transcription of an mRNA is sometimes referred to as the pre-mRNA. After it has been processed and is ready to be exported from the nucleus, it is called the mature mRNA. The three main processing steps for mRNAs are (Figure 7.67):

• Capping at the 5' end
• Splicing to remove introns
• Addition of a polyA tail at the 3' end.

Although this description suggests that these processing steps occur post-transcriptionally, after the entire gene has been transcribed, there is evidence that processing occurs co-transcriptionally. That is, the steps of processing are occurring as the mRNA is being made. Proteins involved in mRNA processing have been shown to be associated with the phosphorylated C-terminal domain (CTD) of RNA polymerase II.

Capping

As might be expected, the addition of an mRNA cap at the 5’ end is the first step in mRNA processing, since the 5’end of the RNA is the first to be made. Capping occurs once the first 20-30 nucleotides of the RNA have been synthesized. The addition of the cap involves removal of a phosphate from the first nucleotide in the RNA to generate a diphosphate. This is then joined to a guanosine monophosphate which is subsequently methylated at N7 of the guanine to form the 7mG cap structure (Figure 7.68). This cap is recognized and bound by a complex of proteins that remain associated with the cap till the mRNA has been transported into the cytoplasm. The cap protects the 5' end of the mRNA from degradation by nucleases and also helps to position the mRNA correctly on the ribosomes during protein synthesis.

Splicing

Eukaryotic genes have introns, noncoding regions that interrupt the gene. The mRNA copied from genes containing introns will also therefore have noncoding regions that interrupt the information in the gene. These noncoding regions must be removed (Figure 7.69) before the mRNA is sent out of the nucleus to be used to direct protein synthesis.

Intron removal

Introns are removed from the pre-mRNA by the activity of a complex called the spliceosome. The spliceosome is made up of proteins and small RNAs that are associated to form protein-RNA enzymes called small nuclear ribonucleoproteins or snRNPs (pronounced snurps).

Splice junctions

The splicing machinery must be able to recognize splice junctions (i.e., where each exon ends and its associated intron begins) in order to correctly cut out the introns and join the exons to make the mature, spliced mRNA. What signals indicate exon-intron boundaries? The junctions between exons and introns are indicated by specific base sequences. The consensus sequence at the 5’ exon-intron junction (also called the 5’ splice site) is AGGURAGU. In this sequence, the intron starts with the second G (R stands for any purine). The 3' splice junction has the consensus sequence YAGRNNN, where YAG is within the intron, and RNNN is part of the exon (Y stands for any pyrimidine, and N for any nucleotide).

There is also a third important sequence within the intron, about a hundred nucleotides from the 3’ splice site, called a branch point or branch site, that is important for splicing. This site is defined by the presence of an A followed by a string of pyrimidines. The

importance of this site will be seen when we consider the steps of splicing.

Splicing mechanism

There are two main steps in splicing. The first step is the nucleophilic attack by the 2'OH of the branch point A on the 5' splice site (the junction of the 5' exon and the intron). As a result of a trans-esterification reaction, the 5' exon is released, and a lariat-shaped molecule composed of the 3' exon and the intron sequence is generated (Figure 7.70). In the second step, the 3' OH of the 5' exon attacks the 3' splice site, and the two exons are joined together, and the lariat-shaped intron is released .

Spliceosome

As mentioned earlier, splicing is carried out by a complex consisting of small RNAs and proteins. The five small RNAs crucial to this complex, U1, U2, U4, U5 and U6 are found associated with proteins, as snRNPs. These and many other proteins work together to facilitate splicing. Although many details remain to be worked out, it appears that components of the splicing machinery associate with the CTD of the RNA polymerase and that this association is important for efficient splicing. The assembly of the spliceosome requires the stepwise interaction of the various snRNPs and other splicing factors (Figure 7.71). The initial step in this process is the interaction of the U1 snRNP with the 5' splice site. Additional proteins such as U2AF (AF = associated factor) are also loaded onto the pre-mRNA near the branch site. This is followed by the binding of the U2 snRNA to the branch site.

Next, a complex of the U4/U6 and U5 snRNPs is recruited to the spliceosome to generate a pre-catalytic complex. This complex undergoes rearrangements that alter RNA-RNA and protein-RNA interactions, resulting in displacement of the U4 and U1 snRNPs and the formation of the catalytically active spliceosome. This complex then carries out the two splicing steps described earlier.

Alternative splicing

On average, human genes have about 9 exons each. However, the mature mRNAs from a gene containing nine exons may not include all of them. This is because the exons in a pre-mRNA can be spliced together in different combinations to generate different mature mRNAs. This is called alternative splicing, and allows the production of many different proteins using relatively few genes, since a single RNA with many exons can, by combining different exons during splicing, create many different protein coding messages. Because of alternative splicing, each gene in our DNA gives rise, on average, to three different proteins. Alternative splicing allows the information in a single gene to be used to specify different proteins in different cell types or at different developmental stages (Figure 7.72).

Polyadenylation

The 3' end of a processed eukaryotic mRNA typically has a "poly(A) tail" consisting of about 200 adenine-containing nucleotides. These residues are added by a template-independent enzyme, poly(A)polymerase, following cleavage of the RNA at a site near the 3' end of the new transcript. Components of the polyadenylation machinery have been shown to be associated with the CTD of the RNA polymerase, showing that all three steps of pre-mRNA processing are tightly linked to transcription. There is evidence that the polyA tail plays a role in efficient translation of the mRNA, as well as in the stability of the mRNA. Like alternative splice sites, genes can have alternative polyA sites as well (Figure 7.73).

The cap and the polyA tail on an mRNA are also indications that the mRNA is complete (i.e., not defective). Once protein-coding messages have been processed by capping, splicing and addition of a poly A tail, they are transported out of the nucleus to be translated in the cytoplasm. Mature mRNAs are sent into the cytoplasm bound to export proteins that interact with the nuclear pore complexes in the nuclear envelope (Figure 7.74). Once the mature mRNA has been translocated to the cytoplasm, it is ready to be translated.

RNA editing

In addition to undergoing the three processing steps outlined above, many RNAs undergo further modification called RNA editing. Editing has been observed in not only mRNAs but also in transfer RNAs and ribosomal RNAs. As the name suggests, RNA editing is a process during which the sequence of the transcript is altered post-transcriptionally. A well-studied example of RNA editing is the alteration of the sequence of the mRNA for apolipoprotein B (see also HERE). The editing results in the deamination of a cytosine in the transcript to form a uracil, at a specific location in the mRNA. This change converts the codon at this position, CAA, which encodes a glutamine, into UAA, a stop codon. The consequence of this is that a shorter version of the protein is made,

when the edited transcript is translated. It is interesting that the editing of this transcript occurs in intestinal cells but not in liver cells. Thus, the protein product of the apolipoprotein B gene is longer in the liver than it is in the intestine.

Insertion/deletion

Another kind of RNA editing involves the insertion or deletion of one or more nucleotides. One example of this sort of editing is seen in the mitochondrial RNAs of trypanosomes. Small guide RNAs indicate the sites at which nucleotides are inserted or deleted to produce the mRNA that is eventually translated (Figure 7.75).

The effect of either of these kinds of editing on the mRNA is that the encoded protein product is different, providing another point at which the product of expression of a gene can be controlled.

tRNA synthesis & processing

tRNAs are synthesized by RNA polymerase III, which makes precursor molecules called pre-tRNA that then undergo processing to generate mature tRNAs. The initial transcripts contain additional RNA sequences at both the 5’ and 3’ ends. Some pre-tRNAs also contain introns. These additional sequences are removed from the transcript during processing.

The 5’ leader sequence of the pre-tRNA (the additional nucleotides at the 5’-end) is removed by an unusual endonuclease called ribonuclease P (RNase P - Figure 7.76). RNase is a ribonucleoprotein complex composed of a catalytic RNA and numerous proteins. The 3’ trailer sequence (extra nucleotides at the 3’ end of the pre-tRNA) is later removed by different nucleases. All tRNAs must have a 3’ CCA sequence that is necessary for the charging of the tRNAs with amino acids. In bacteria, this CCA sequence is encoded in the tRNA gene, but in eukaryotes, the CCA sequence is added post-transcriptionally by an enzyme called tRNA nucleotidyl transferase (tRNT).

Introns

As mentioned earlier, some tRNA precursors contain an intron located in the anticodon arm. In eukaryotes, this intron is typically found immediately 3’ to the anticodon. The introns is spliced out with the help of a tRNA splicing endonuclease and a ligase.

Base modifications

Mature tRNAs contain a high proportion of bases other than the usual adenine (A), guanine (G), cytidine (C) and uracil (U). These unusual bases are produced by modifying the bases in the tRNA to form variants, such as pseudouridine (Figure 7.77) or dihyrouridine. Modifications to the bases are introduced into the tRNA at the final processing step by a variety of specialized enzymes. Different tRNAs have different subsets of modifications at specific locations, often the first base of the anti-codon (the wobble position).

rRNA synthesis and processing

Cells contain many copies of rRNA genes (between 100 and 2000 copies are seen in mammalian cells). These genes are organized in transcription units separated by non-transcribed spacers. Each transcription unit contains sequences coding for 18S, 5.8S and 28S rRNA, and is transcribed by RNA polymerase I into a single long transcript (47S). The 5S rRNA is separately transcribed. The sizes of ribosomal RNAs are, by convention, indicated by their sedimentation coefficients, which is a measure of their rate of sedimentation during centrifugation. Sedimentation is expressed in Svedberg units (hence the S at the end of the number) with larger numbers indicating greater mass.

The initial transcript contains 5’ and 3’ external transcribed spacers (ETS) as well as internal transcribed sequences (ITS). The primary transcript is first trimmed at both ends by nucleases to give a 45S pre-rRNA. Further processing of the pre-rRNA through cleavages guided by RNA-protein complexes containing snoRNAs (small nucleolar RNAs), gives rise to the mature 18S, 5.8S and 28S rRNAs (Figure 7.79). Ribosomal RNAs are also modified both on the ribose sugars and on the bases. Interestingly, methylation of ribose sugars is the major modification in rRNA. The modified base pseudouridine is also common in rRNA. Other modifications include base methylation, and acetylation. These modifications are thought to be important in modulating ribosome function.

YouTube Lectures

by Kevin

HERE & HERE

768

Figure 7.68 - 5’ capping of eukaryotic mRNAs

Wikipedia

Figure 7.67 - Steps in processing of pre-mRNA

769

Figure 7.69 - Removal of introns from the primary transcript

Interactive Learning

Module

HERE

770

Figure 7.70 - Splicing of introns

Wikipedia

771

Figure 7.71 - Assembly of the spliceosome complex

Wikipedia

YouTube Lectures

by Kevin

HERE & HERE

772

Figure 7.72 - Alternative splicing leads to different forms of a protein from the same gene sequence

Figure 7.73 - Alternative poly-adenylation sites for a gene

773

Figure 7.74 - Structure of a mature eukaryotic mRNA

Interactive Learning

Module

HERE

774

Figure 7.76 - Structure of the RNA component of ribonuclease P

Figure 7.75 - Template guided - one mechanism of RNA editing

775

Figure 7.78 - Sequence of a mature tRNA

Wikipedia

Figure 7.77 - Synthesis of pseudouridine from uridine

Wikipedia

776

Figure 7.79 - Processing of ribosomal RNA

YouTube Lectures

by Kevin

HERE & HERE

Graphic images in this book were products of the work of several talented students. Links to their Web pages are below

Click HERE for

Martha Baker's

Web Page

Click HERE for

Pehr Jacobson's

Web Page

Click HERE for

Aleia Kim's

Web Page

Click HERE for

Penelope Irving's

Web Page

Problem set related to this section HERE

Point by Point summary of this section HERE

To get a certificate for mastering this section of the book, click HERE

Kevin Ahern's free iTunes U Courses - Basic / Med School / Advanced

Biochemistry Free & Easy (our other book) HERE / Facebook Page

Kevin and Indira's Guide to Getting into Medical School - iTunes U Course / Book

To see Kevin Ahern's OSU ecampus courses - BB 350 / BB 450 / BB 451

To register for Kevin Ahern's OSU ecampus courses - BB 350 / BB 450 / BB 451

Biochemistry Free For All Facebook Page (please like us)

Kevin Ahern's Web Page / Facebook Page / Taralyn Tan's Web Page

Kevin Ahern's free downloads HERE

OSU's Biochemistry/Biophysics program HERE

OSU's College of Science HERE

Oregon State University HERE

Email Kevin Ahern / Indira Rajagopal / Taralyn Tan

778

The Codon Song

To the tune of "When I'm Sixty Four"

Metabolic Melodies Website HERE

Building of proteins, you oughta know
Needs amino A's
Peptide bond catalysis in ribosomes
Triplet bases, three letter codes

Mixing and matching nucleotides
Who is keeping score?
Here is the low down
If you count codons
You'll get sixty four

Got - to - line - up - right
16-S R-N-A and
Shine Dalgarno site

You can make peptides, every size
With the proper code
Start codons positioned
In the P site place
Initiator t-RNAs

UGA stops and AUGs go
Who could ask for more?
You know the low down
Count up the codons
There are sixty four

Recording by Tim Karplus

Lyrics by Kevin Ahern
Recording by Tim Karplus Lyrics by Kevin Ahern

This page titled 4.6.1: RNA Processing is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

4.7: RNA Processing

Source: BiochemFFA_7_5.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

So far, we have looked at the mechanism by which the information in genes (DNA) is transcribed into RNA. The newly made RNA, also known as the primary transcript is further processed before it is functional. Both prokaryotes and eukaryotes process their ribosomal and transfer RNAs. The major difference in RNA processing, however, between prokaryotes and eukaryotes, is in the processing of messenger RNAs. We will focus on the processing of mRNAs in this section. You will recall that in bacterial cells, the mRNA is translated directly as it comes off the DNA template. In eukaryotic cells, RNA synthesis, which occurs in the nucleus, is separated from the protein synthesis machinery, which is in the cytoplasm. The initial product of transcription of an mRNA is sometimes referred to as the pre-mRNA. After it has been processed and is ready to be exported from the nucleus, it is called the mature mRNA. The three main processing steps for mRNAs are (Figure 7.67):

• Capping at the 5' end
• Splicing to remove introns
• Addition of a polyA tail at the 3' end.

Although this description suggests that these processing steps occur post-transcriptionally, after the entire gene has been transcribed, there is evidence that processing occurs co-transcriptionally. That is, the steps of processing are occurring as the mRNA is being made. Proteins involved in mRNA processing have been shown to be associated with the phosphorylated C-terminal domain (CTD) of RNA polymerase II.

Capping

As might be expected, the addition of an mRNA cap at the 5’ end is the first step in mRNA processing, since the 5’end of the RNA is the first to be made. Capping occurs once the first 20-30 nucleotides of the RNA have been synthesized. The addition of the cap involves removal of a phosphate from the first nucleotide in the RNA to generate a diphosphate. This is then joined to a guanosine monophosphate which is subsequently methylated at N7 of the guanine to form the 7mG cap structure (Figure 7.68). This cap is recognized and bound by a complex of proteins that remain associated with the cap till the mRNA has been transported into the cytoplasm. The cap protects the 5' end of the mRNA from degradation by nucleases and also helps to position the mRNA correctly on the ribosomes during protein synthesis.

Splicing

Eukaryotic genes have introns, noncoding regions that interrupt the gene. The mRNA copied from genes containing introns will also therefore have noncoding regions that interrupt the information in the gene. These noncoding regions must be removed (Figure 7.69) before the mRNA is sent out of the nucleus to be used to direct protein synthesis.

Intron removal

Introns are removed from the pre-mRNA by the activity of a complex called the spliceosome. The spliceosome is made up of proteins and small RNAs that are associated to form protein-RNA enzymes called small nuclear ribonucleoproteins or snRNPs (pronounced snurps).

Splice junctions

The splicing machinery must be able to recognize splice junctions (i.e., where each exon ends and its associated intron begins) in order to correctly cut out the introns and join the exons to make the mature, spliced mRNA. What signals indicate exon-intron boundaries? The junctions between exons and introns are indicated by specific base sequences. The consensus sequence at the 5’ exon-intron junction (also called the 5’ splice site) is AGGURAGU. In this sequence, the intron starts with the second G (R stands for any purine). The 3' splice junction has the consensus sequence YAGRNNN, where YAG is within the intron, and RNNN is part of the exon (Y stands for any pyrimidine, and N for any nucleotide).

There is also a third important sequence within the intron, about a hundred nucleotides from the 3’ splice site, called a branch point or branch site, that is important for splicing. This site is defined by the presence of an A followed by a string of pyrimidines. The

importance of this site will be seen when we consider the steps of splicing.

Splicing mechanism

There are two main steps in splicing. The first step is the nucleophilic attack by the 2'OH of the branch point A on the 5' splice site (the junction of the 5' exon and the intron). As a result of a trans-esterification reaction, the 5' exon is released, and a lariat-shaped molecule composed of the 3' exon and the intron sequence is generated (Figure 7.70). In the second step, the 3' OH of the 5' exon attacks the 3' splice site, and the two exons are joined together, and the lariat-shaped intron is released .

Spliceosome

As mentioned earlier, splicing is carried out by a complex consisting of small RNAs and proteins. The five small RNAs crucial to this complex, U1, U2, U4, U5 and U6 are found associated with proteins, as snRNPs. These and many other proteins work together to facilitate splicing. Although many details remain to be worked out, it appears that components of the splicing machinery associate with the CTD of the RNA polymerase and that this association is important for efficient splicing. The assembly of the spliceosome requires the stepwise interaction of the various snRNPs and other splicing factors (Figure 7.71). The initial step in this process is the interaction of the U1 snRNP with the 5' splice site. Additional proteins such as U2AF (AF = associated factor) are also loaded onto the pre-mRNA near the branch site. This is followed by the binding of the U2 snRNA to the branch site.

Next, a complex of the U4/U6 and U5 snRNPs is recruited to the spliceosome to generate a pre-catalytic complex. This complex undergoes rearrangements that alter RNA-RNA and protein-RNA interactions, resulting in displacement of the U4 and U1 snRNPs and the formation of the catalytically active spliceosome. This complex then carries out the two splicing steps described earlier.

Alternative splicing

On average, human genes have about 9 exons each. However, the mature mRNAs from a gene containing nine exons may not include all of them. This is because the exons in a pre-mRNA can be spliced together in different combinations to generate different mature mRNAs. This is called alternative splicing, and allows the production of many different proteins using relatively few genes, since a single RNA with many exons can, by combining different exons during splicing, create many different protein coding messages. Because of alternative splicing, each gene in our DNA gives rise, on average, to three different proteins. Alternative splicing allows the information in a single gene to be used to specify different proteins in different cell types or at different developmental stages (Figure 7.72).

Polyadenylation

The 3' end of a processed eukaryotic mRNA typically has a "poly(A) tail" consisting of about 200 adenine-containing nucleotides. These residues are added by a template-independent enzyme, poly(A)polymerase, following cleavage of the RNA at a site near the 3' end of the new transcript. Components of the polyadenylation machinery have been shown to be associated with the CTD of the RNA polymerase, showing that all three steps of pre-mRNA processing are tightly linked to transcription. There is evidence that the polyA tail plays a role in efficient translation of the mRNA, as well as in the stability of the mRNA. Like alternative splice sites, genes can have alternative polyA sites as well (Figure 7.73).

The cap and the polyA tail on an mRNA are also indications that the mRNA is complete (i.e., not defective). Once protein-coding messages have been processed by capping, splicing and addition of a poly A tail, they are transported out of the nucleus to be translated in the cytoplasm. Mature mRNAs are sent into the cytoplasm bound to export proteins that interact with the nuclear pore complexes in the nuclear envelope (Figure 7.74). Once the mature mRNA has been translocated to the cytoplasm, it is ready to be translated.

RNA editing

In addition to undergoing the three processing steps outlined above, many RNAs undergo further modification called RNA editing. Editing has been observed in not only mRNAs but also in transfer RNAs and ribosomal RNAs. As the name suggests, RNA editing is a process during which the sequence of the transcript is altered post-transcriptionally. A well-studied example of RNA editing is the alteration of the sequence of the mRNA for apolipoprotein B (see also HERE). The editing results in the deamination of a cytosine in the transcript to form a uracil, at a specific location in the mRNA. This change converts the codon at this position, CAA, which encodes a glutamine, into UAA, a stop codon. The consequence of this is that a shorter version of the protein is made,

when the edited transcript is translated. It is interesting that the editing of this transcript occurs in intestinal cells but not in liver cells. Thus, the protein product of the apolipoprotein B gene is longer in the liver than it is in the intestine.

Insertion/deletion

Another kind of RNA editing involves the insertion or deletion of one or more nucleotides. One example of this sort of editing is seen in the mitochondrial RNAs of trypanosomes. Small guide RNAs indicate the sites at which nucleotides are inserted or deleted to produce the mRNA that is eventually translated (Figure 7.75).

The effect of either of these kinds of editing on the mRNA is that the encoded protein product is different, providing another point at which the product of expression of a gene can be controlled.

tRNA synthesis & processing

tRNAs are synthesized by RNA polymerase III, which makes precursor molecules called pre-tRNA that then undergo processing to generate mature tRNAs. The initial transcripts contain additional RNA sequences at both the 5’ and 3’ ends. Some pre-tRNAs also contain introns. These additional sequences are removed from the transcript during processing.

The 5’ leader sequence of the pre-tRNA (the additional nucleotides at the 5’-end) is removed by an unusual endonuclease called ribonuclease P (RNase P - Figure 7.76). RNase is a ribonucleoprotein complex composed of a catalytic RNA and numerous proteins. The 3’ trailer sequence (extra nucleotides at the 3’ end of the pre-tRNA) is later removed by different nucleases. All tRNAs must have a 3’ CCA sequence that is necessary for the charging of the tRNAs with amino acids. In bacteria, this CCA sequence is encoded in the tRNA gene, but in eukaryotes, the CCA sequence is added post-transcriptionally by an enzyme called tRNA nucleotidyl transferase (tRNT).

Introns

As mentioned earlier, some tRNA precursors contain an intron located in the anticodon arm. In eukaryotes, this intron is typically found immediately 3’ to the anticodon. The introns is spliced out with the help of a tRNA splicing endonuclease and a ligase.

Base modifications

Mature tRNAs contain a high proportion of bases other than the usual adenine (A), guanine (G), cytidine (C) and uracil (U). These unusual bases are produced by modifying the bases in the tRNA to form variants, such as pseudouridine (Figure 7.77) or dihyrouridine. Modifications to the bases are introduced into the tRNA at the final processing step by a variety of specialized enzymes. Different tRNAs have different subsets of modifications at specific locations, often the first base of the anti-codon (the wobble position).

rRNA synthesis and processing

Cells contain many copies of rRNA genes (between 100 and 2000 copies are seen in mammalian cells). These genes are organized in transcription units separated by non-transcribed spacers. Each transcription unit contains sequences coding for 18S, 5.8S and 28S rRNA, and is transcribed by RNA polymerase I into a single long transcript (47S). The 5S rRNA is separately transcribed. The sizes of ribosomal RNAs are, by convention, indicated by their sedimentation coefficients, which is a measure of their rate of sedimentation during centrifugation. Sedimentation is expressed in Svedberg units (hence the S at the end of the number) with larger numbers indicating greater mass.

The initial transcript contains 5’ and 3’ external transcribed spacers (ETS) as well as internal transcribed sequences (ITS). The primary transcript is first trimmed at both ends by nucleases to give a 45S pre-rRNA. Further processing of the pre-rRNA through cleavages guided by RNA-protein complexes containing snoRNAs (small nucleolar RNAs), gives rise to the mature 18S, 5.8S and 28S rRNAs (Figure 7.79). Ribosomal RNAs are also modified both on the ribose sugars and on the bases. Interestingly, methylation of ribose sugars is the major modification in rRNA. The modified base pseudouridine is also common in rRNA. Other modifications include base methylation, and acetylation. These modifications are thought to be important in modulating ribosome function.

YouTube Lectures

by Kevin

HERE & HERE

768

Figure 7.68 - 5’ capping of eukaryotic mRNAs

Wikipedia

Figure 7.67 - Steps in processing of pre-mRNA

769

Figure 7.69 - Removal of introns from the primary transcript

Interactive Learning

Module

HERE

770

Figure 7.70 - Splicing of introns

Wikipedia

771

Figure 7.71 - Assembly of the spliceosome complex

Wikipedia

YouTube Lectures

by Kevin

HERE & HERE

772

Figure 7.72 - Alternative splicing leads to different forms of a protein from the same gene sequence

Figure 7.73 - Alternative poly-adenylation sites for a gene

773

Figure 7.74 - Structure of a mature eukaryotic mRNA

Interactive Learning

Module

HERE

774

Figure 7.76 - Structure of the RNA component of ribonuclease P

Figure 7.75 - Template guided - one mechanism of RNA editing

775

Figure 7.78 - Sequence of a mature tRNA

Wikipedia

Figure 7.77 - Synthesis of pseudouridine from uridine

Wikipedia

776

Figure 7.79 - Processing of ribosomal RNA

YouTube Lectures

by Kevin

HERE & HERE

Graphic images in this book were products of the work of several talented students. Links to their Web pages are below

Click HERE for

Martha Baker's

Web Page

Click HERE for

Pehr Jacobson's

Web Page

Click HERE for

Aleia Kim's

Web Page

Click HERE for

Penelope Irving's

Web Page

Problem set related to this section HERE

Point by Point summary of this section HERE

To get a certificate for mastering this section of the book, click HERE

Kevin Ahern's free iTunes U Courses - Basic / Med School / Advanced

Biochemistry Free & Easy (our other book) HERE / Facebook Page

Kevin and Indira's Guide to Getting into Medical School - iTunes U Course / Book

To see Kevin Ahern's OSU ecampus courses - BB 350 / BB 450 / BB 451

To register for Kevin Ahern's OSU ecampus courses - BB 350 / BB 450 / BB 451

Biochemistry Free For All Facebook Page (please like us)

Kevin Ahern's Web Page / Facebook Page / Taralyn Tan's Web Page

Kevin Ahern's free downloads HERE

OSU's Biochemistry/Biophysics program HERE

OSU's College of Science HERE

Oregon State University HERE

Email Kevin Ahern / Indira Rajagopal / Taralyn Tan

778

The Codon Song

To the tune of "When I'm Sixty Four"

Metabolic Melodies Website HERE

Building of proteins, you oughta know
Needs amino A's
Peptide bond catalysis in ribosomes
Triplet bases, three letter codes

Mixing and matching nucleotides
Who is keeping score?
Here is the low down
If you count codons
You'll get sixty four

Got - to - line - up - right
16-S R-N-A and
Shine Dalgarno site

You can make peptides, every size
With the proper code
Start codons positioned
In the P site place
Initiator t-RNAs

UGA stops and AUGs go
Who could ask for more?
You know the low down
Count up the codons
There are sixty four

Recording by Tim Karplus

Lyrics by Kevin Ahern
Recording by Tim Karplus Lyrics by Kevin Ahern

This page titled 4.7: RNA Processing is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

4.8: Translation

Source: BiochemFFA_7_6.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

Translation is the process by which information in mRNAs is used to direct the synthesis of proteins. As you have learned in introductory biology, in eukaryotic cells, this process is carried out in the cytoplasm of the cell, by large RNA-protein machines called ribosomes. Ribosomes contain ribosomal RNA (rRNA) and proteins. The proteins and rRNA are organized into two subunits, a large and a small. Ribosomes function by binding to mRNAs and holding them in a way that allows the amino acids encoded by the RNA to be joined sequentially to form a polypeptide. Transfer RNAs are the carriers of the appropriate amino acids to the ribosome.

The Genetic Code

We speak of genes (i.e., DNA) coding for proteins and the central dogma, which states that DNA makes RNA makes protein. What does this actually mean? A code can be thought of as a system for storing or communicating information. A familiar example is the use of letters to represent the names of airports (e.g., PDX for Portland, Oregon and ORD for Chicago's O'Hare). When a tag on your luggage shows PDX as the destination, it conveys information that your bag should be sent to Portland, Oregon. To function well, such a set-up must have unique identifiers for each airport and people who can decode the identifiers correctly. That is, PDX must stand only for Portland, Oregon and no other airport. Also, luggage handlers must be able to correctly recognize what PDX stands for, so that your luggage doesn't land in Phoenix, instead.

How does this relate to genes and the proteins they encode?

Genes are first transcribed into mRNA, as we have already discussed. The sequence of an mRNA, copied from a gene, directly specifies the sequence of amino acids in the protein it encodes. Each amino acid in the protein is specified by a sequence of 3 bases called a codon in the mRNA (Figure 7.81). For example, the amino acid tryptophan is encoded by the sequence UGG on an mRNA. All of the twenty amino acids used to build proteins have, likewise, 3-base sequences that encode them.

Degeneracy

Given that there are 4 bases in RNA, the number of different 3-base combinations that are possible is 43, or 64. There are, however, only 20 amino acids that are used in building proteins in cells. This discrepancy in the number of possible codons and the actual number of amino acids they specify is explained by the fact that the same amino acid may be specified by more than one codon. In fact, with the exception of the amino acids methionine and tryptophan, all the other amino acids are encoded by multiple codons. Codons for the same amino acid are often related, with the first two bases the same and the third being variable. An example would be the codons for alanine: GCU, GCA, GCC and GCG all stand for alanine. This sort of redundancy in the genetic code is termed degeneracy.

Stop and start codons

Three of the 64 codons are what are known as termination or stop codons, and as their name suggests, indicate the end of a protein coding sequence. The codon for methionine, AUG, is used as the initiation or start codon for the majority of proteins. This ingenious system is used to direct the assembly of a protein in the same way that you might string together colored beads in a particular order using instructions that used symbols like UGG for a red bead, followed by UUU for a green bead, CAC for yellow, and so on, till you came to UGA, indicating that you should stop stringing beads.

Translating the code

While the ribosomes are literally the factories that join amino acids together using the instructions in mRNAs, another class of RNA molecules, the transfer RNAs (tRNAs) are also needed for translation (Figure 7.83 and Interactive 7.1). Transfer RNAs are small RNA molecules, about 75-90 nucleotides long, that function to 'interpret' the instructions in the mRNA during protein synthesis. Transfer RNAs are extensively modified post-transcriptionally and contain a large number of unusual bases. The sequences of tRNAs have several self complementary regions, where the single-stranded tRNA folds on itself and base-pairs to form what is sometimes described as a clover leaf structure.

This structure is crucial to the function of the tRNA, providing both the sites for attachment of the appropriate amino acid and for recognition of codons in the mRNA. In terms of the bead analogy above, someone or something has to be able to bring a red bead in when the instructions indicate UGG, and a green bead when the instructions say UUU. This, then, is the function of the tRNAs. They must be able to bring the amino acid corresponding to the instructions to the ribosome.

t-RNA specificity

A given transfer RNA is specific for a particular amino acid. It is linked covalently at its 3' end to the appropriate amino acid by an enzyme called aminoacyl tRNA synthetase. For example, there is a transfer RNA that is specific to the amino acid tryptophan, and a corresponding aminoacyl tRNA synthetase, called a tryptophanyl tRNA synthetase, that can attach the tryptophan specifically to this tRNA. Likewise, there is an aminoacyl tRNA synthetase specific for each amino acid. A tRNA with an amino acid attached to it is said to be charged (Figure 7.84). A pool of charged tRNAs is necessary to carry out protein synthesis. How do these tRNAs, carrying specific amino acids assist the ribosome in stringing together the correct amino acids, as specified by the sequence of the mRNA?

Codon recognition

As we already know, the amino acid sequence of the protein is determined by the order of the codons in the mRNA. We also have charged tRNAs carrying the various amino acids present.

How are the amino acids attached to each other in the order indicated by the base sequence of the mRNA? This requires recognition of the codons on the mRNA by the appropriate charged tRNAs. The amino acid tryptophan, as we noted, is specified by the codon UGG in the mRNA. This codon must be recognized by a tRNA charged with tryptophan. Every tRNA has a a sequence of 3 bases, the anticodon, that is complementary to the codon for the amino acid it is carrying. When the tRNA encounters the codon for its amino acid on the messenger RNA, the anticodon will base-pair with the codon. For the tryptophan tRNA this is what it would look like:

Sequence of tryptophan codon in mRNA:

5’ UGG 3’

Anticodon sequence in tryptophan tRNA:

5’ CCA 3’

Note that the sequences are both written, by convention, in the 5’ to 3’ direction. To base pair, though, they must be oriented in opposite directions (anti-parallel). The codon-anticodon basepair in the antiparallel orientation then would be:

5’ UGG 3’
3’ ACC 5’

The base-pairing of the anticodon on a charged tRNA with the codon on the mRNA is what brings the correct amino acids in to the ribosome to be added on to the growing protein chain (Figure 7.85).

Making a Polypeptide

With an idea of the various components necessary for translation and how they work, we can now take a look at the process of protein synthesis. The main steps in the process are similar in prokaryotes and eukaryotes. As we already noted, ribosomes bind to mRNAs and facilitate the interaction between the codons in the mRNA and the anticodons on charged tRNAs.

Ribosomes have two sites (P-site and A-site) for binding and positioning charged tRNAs so each can form base pairs between their anticodon and a codon from the mRNA. The start codon (AUG) is positioned to base pair with the tRNA in the P-site (peptidyl site). Next, the charged tRNA complementary to the codon adjacent to the start codon binds and occupies the A-site (aminoacyl site) in the ribosome (Figure 7.86).

At this point, the ribosome joins the amino acids carried on each tRNA by making a peptide bond. The bond between the amino acid and the tRNA in the P-site is broken and the dipeptide is joined to the tRNA on the A-site.

The initiator tRNA without its amino acid is then released, moving into a site known as the Exit or E-site, while the second tRNA carrying the dipeptide (and the codon it is base paired to) moves into the P-site. The A-site now is ready with a new codon for the

next incoming charged tRNA. This process is repeated, with the ribosome moving on the mRNA one codon at a time, until the stop codon reaches the A-site. At this point, a release factor binds at the A-site, and helps to free the completed polypeptide from the ribosome. The ribosome then dissociates into the small and large subunits, once more.

Three steps

Having considered the steps of translation in broader terms, we can now look at them in greater detail. We will consider the three steps of translation (bel0w) individually.

Initiation (binding of the ribosomal subunits to the transcript and initiator tRNA)
Elongation (repeated addition of amino acids to the growing polypeptide, based on the sequence of the mRNA - Figure 7.87)
Termination (release of the completed polypeptide and dissociation of the ribosome into its subunits).

We already know that processed mRNAs are sent from the nucleus to the cytoplasm in eukaryotic cells, while in prokaryotic cells, transcription and translation occur in a single cellular compartment. The small and large subunits of ribosomes, each composed of characteristic rRNAs and proteins, are found in the cytoplasm and assemble on mRNAs to form complete ribosomes that carry out translation. Both prokaryotic and eukaryotic ribosomal subunits are made up of one or more major rRNAs together with a large number of ribosomal proteins. The small subunits of prokaryotic cells are called the 30S ribosomal subunits, while their counterparts in eukaryotes are the 40S subunits. The large ribosomal subunits in prokaryotes are the 50S subunits, while those in eukaryotic cells are 60S. These differences reflect the larger mass of eukaryotic ribosomes. The rRNA components of ribosomes are important for the recognition of the 5’ end of the mRNA, and also play a catalytic role in the formation of peptide bonds.

Initiation

Messenger RNAs have non-coding sequences both at their 5' and 3' ends, with the actual protein-coding region sandwiched in between these untranslated regions (called the 5' UTR and 3' UTR, respectively). The ribosome must be able to recognize the 5' end of the mRNA and bind to it, then determine where the start codon is located. It is important to note that both in prokaryotes and eukaryotes, ribosomes assemble at the 5’ end of the transcript by the stepwise binding of the small and large subunits. The small subunit first binds to the mRNA at specific sequences in the 5’ UTR. The large subunit then binds to the complex of the mRNA and small subunit, to form the complete ribosome.

Initiator tRNA

Initiation also requires the binding of the first tRNA to the ribosome. As we have noted earlier, the initiation, or start codon is usually AUG, which codes for the amino acid methionine. Thus, the initiator tRNA is one that carries methionine and is designated as tRNAmet or methionyl tRNAmet. In bacteria, the methionine on the initiator tRNA is modified by the addition of a formyl group, and is designated tRNAfmet. The initiator tRNA carrying methionine to the AUG is different from the tRNAs that carry methionine intended for other positions in proteins. As such, the initiator tRNA is sometimes referred to as tRNAimet.

Prokaryotic initiation

In prokaryotes, the 5’ end of the mRNA is the only free end available, as transcription is tightly coupled to translation and the entire mRNA is not transcribed before translation begins. Nevertheless, the ribosome must be correctly positioned at the 5’ end of the messenger RNA in order to initiate translation. How does the ribosome “know” exactly where to bind in the 5’UTR of the mRNA?

Shine-Dalgarno sequence

Examination of the sequences upstream of the start codon in prokaryotic mRNAs reveals that there is a short purine-rich sequence ahead of the start codon that is crucial to recognition and binding by the small ribosomal subunit (Figure 7.89). This sequence, called the Shine-Dalgarno sequence, is complementary to a stretch of pyrimidines at the 3’ end of the 16S rRNA component of the small ribosomal subunit (Figure 7.90). Base-pairing between these complementary sequences positions the small ribosomal subunit at the right spot on the mRNA, with the AUG start codon at the P-site.

Initiation factors

The binding of the small ribosomal subunit to the mRNA requires the assistance of three protein factors called Initiation Factors 1, 2 and 3 (IF1, IF2, IF3). These proteins, which are associated with the small ribosomal subunit, are necessary for its binding to mRNA, but dissociate from it when the 50S ribosomal subunit binds. Of these proteins, IF3 is important for the binding of the

small subunit to the mRNA, while IF2 is involved in bringing the initiator tRNA to the partial P-site of the small ribosomal subunit. IF1 occupies the A-site, preventing the binding of the initiator tRNA at that site.

Once the small ribosomal subunit is bound to the mRNA and the initiator tRNA is positioned at the P-site, the large ribosomal subunit is recruited and the initiation complex is formed. Binding of the 50S ribosomal subunit is accompanied by the dissociation of all three initiation factors. The removal of IF1 from the A-site on the ribosome frees up the site for the binding of the charged tRNA corresponding to the second codon (Figure 7.91).

Eukaryotic initiation

In eukaryotes, initiation follows a similar pattern, although the order of events and the specific initiation factors are different.

Eukaryotes have a large number of IFs that are known as eIFs (eukaryotic initiation factors). These initiation factors are involved in the binding of the initiator tRNA to the small subunit, as well as in association of the small subunit with mRNA and subsequent attachment of the large subunit.

Ribosome assembly

The assembly of the translation machinery in eukaryotes begins with the binding of the initiator tRNA to the 40S (small) subunit. This step requires the assistance of eIF2 and eIF3. Next the small subunit with the initiator tRNA binds to the 7-methyl G cap on the 5'end of the mRNA. The 40S subunit then moves along the mRNA, scanning for a a start codon. Binding of the ribosomal subunit to the mRNA is dependent not just on finding an AUG, but on the sequences surrounding the codon.

Kozak sequences

Specific sequences surrounding the AUG, called Kozak sequences for the scientist who defined them, have been shown to be necessary for the binding of the 40S subunit, with the bases at -4 and +1 relative to the AUG being especially important (Figure 7.92). Once the small subunit is properly positioned, the large ribosomal subunit (60S) binds, forming the initiation complex.

Elongation

After the ribosome is assembled with the initiator tRNA positioned at the AUG in the P-site, the addition of further amino acids can begin. In both prokaryotes and eukaryotes, the elongation of the polypeptide chain requires the assistance of elongation factors. In bacteria, the binding of the second charged tRNA at the A-site requires the elongation factor EF-Tu complexed with GTP (Figure 7.93). When the charged tRNA has been loaded at the A-site, EF-Tu hydrolyzes the GTP to GDP and dissociates from the ribosome. The free EF-Tu can then work with another charged tRNA to help position it at the A-site (Figure 7.94), after exchanging its GDP for a new GTP.

The corresponding step in eukaryotic cells is dependent on the elongation factor eEF1α.GTP. Once both P-site and A-site are occupied, the methionine carried by the tRNA in the P-site is joined to the amino acid carried by tRNA in the A-site, forming a peptide bond.

The reaction that joins the amino acids occurs in the ribosomal peptidyl transferase center, which is part of the large ribosomal subunit (Figure 7.95).

Ribozyme

Interestingly, there is strong evidence that this reaction is catalyzed by rRNA components of the large subunit, making the formation of peptide bonds an example of the activity of RNA enzymes, or ribozymes. The result of the peptidyl transferase activity is that the tRNA in the A-site now has two amino acids attached to it, while the tRNA at the P-site has none. This “empty” or deacylated tRNA is moved to the E-site on the ribosome, from which it can exit. The tRNA in the A-site, then moves to occupy the vacated P-site, leaving the A-site open for the next incoming charged tRNA. Yet another elongation factor, EF-G complexed with GTP, is required for the translocation of the ribosome along the mRNA in bacteria, while in eukaryotes, this role is played by eEF2.GTP. Repeated cycles of these steps result in the elongation of the polypeptide by one amino acid per cycle, until a termination, or stop codon is in the A-site.

Termination

When a stop codon is in the A-site, proteins called release factors (RFs) are needed to recognize the stop codon and cleave and release the newly made polypeptide. In bacteria, RF1 is a release factor that can recognize the stop codon UAG, while RF2

recognizes UGA. Both RF1 and RF2 can recognize UAA. A third release factor, RF3, works with RF1 and RF2 to hydrolyze the linkage between the polypeptide and the final tRNA, to release the newly synthesized protein. This is followed by the dissociation of the ribosomal subunits from the mRNA, ending the process of translation .

Polypeptide processing

What happens to the newly synthesized polypeptide after it is released from the ribosome? As you know, functional proteins are not simply strings of amino acids. The polypeptide must fold properly in order to perform its function in the cell. It may also undergo a variety of modifications such as the addition of phosphate groups, sugars, lipids, etc. Some proteins are produced as inactive precursors that must be cleaved by proteases to be functional.

An additional challenge in eukaryotic cells is the presence of internal, membrane-bounded compartments. Each compartment contains different proteins with different functions. But the vast majority of proteins in eukaryotic cells are made by ribosomes, free or membrane-bound, in the cytoplasm of the cell (the exceptions are a handful of proteins made within mitochondria and chloroplasts).

Delivery

Each of the thousands of proteins made in the cytoplasm must, therefore, be delivered to the appropriate cellular compartment in which it functions. Some proteins are delivered to their destinations in an unfolded state, and are folded within the compartment in which they function. Others are fully folded and may be post-translationally modified before they are sent to their cellular (or extracellular) destinations.

Some proteins are delivered as they are being synthesized (co-translationally - see Movie 7.3) while others are sorted to their compartments post-translationally. But, with the exception of cytosolic proteins, all proteins must cross membrane barriers, through membrane channels or other "gates", or by transport within membrane vesicles that fuse with the membrane of the target organelle to deliver their contents.

Folding and post-translational modifications

Proper folding of a protein into its 3-dimensional conformation is necessary for it to function effectively. As described in an earlier chapter (HERE), the folding of a protein is largely influenced by hydrophobic interactions that result in folding of the protein in such a way as to position hydrophobic residues in the interior, or core, of the protein, away from the aqueous environment of the cell.

Proper folding may also involve the interaction of regions of the polypeptide that are distant from each other, so that portions of the N-terminal region of the polypeptide may be in close proximity to parts of the C-terminus of the final folded molecule.

As a polypeptide emerges from the ribosome, however, the N-terminal region of the polypeptide may begin to fold on itself, with adjacent parts of the chain interacting in inappropriate ways, before the entire protein has been made. This can result in misfolding of the protein and consequent malfunction. To prevent misfolding, cells have protein chaperones, whose function is to bind to and shield regions of polypeptides as they emerge from the ribosome, and keep them from improperly interacting with one another or with other proteins in the vicinity, until they can fold into their correct final shape (Figure 7.98). In addition, there are classes of chaperones that are able sequester proteins in such a way as to permit unfolding and refolding of misfolded polypeptides. These proteins ensure that the vast majority of proteins in cells are folded into their correct, functional 3-dimensional shapes.

Protein sorting

The process by which proteins are identified as belonging to a particular compartment and then correctly delivered to that destination is known as protein sorting. How does a cell know where a particular protein should be sent?

Proteins have "address labels" or sorting signals that indicate which cellular compartment they are destined for. Characteristic sorting signals are found on proteins that are sent to the nucleus, the ER (Figure 7.99), the mitochondria, etc.

Signal sequences

What do these sorting signals look like?
Most sorting signals (also called signal sequences) are short stretches of amino acid sequence (that is, they are part of the amino acid sequence of the protein). Different cellular compartments have different "address labels".

Signal sequences may be found at the N-terminal or C-terminal region of proteins, or they may be within the amino acid sequence of the proteins. The location of the signal sequence for any given protein is fixed, however. Signal sequences for proteins to be delivered to the endoplasmic reticulum (ER) are found at the N-terminus of the protein. Mitochondrial and chloroplast proteins encoded by nuclear genes also have N-terminal signal sequences. Signal sequences for nuclear proteins, by contrast, are internal to the polypeptide, and may consist of one or more stretches of amino acids that will be displayed on the surface of these proteins once they are folded.

Free and membrane-bound ribosomes

Proteins are synthesized by ribosomes in the cytoplasm or by those that associate with membranes temporarily (membrane-bound ribosomes). The free ribosomes make proteins that are destined for the nucleus, as well as those going to chloroplasts, mitochondria and peroxisomes. Nuclear proteins are delivered in their folded state, while chloroplast and mitochondrial proteins are threaded through translocation channels in the membranes of these organelles, to be folded at their destination.

Proteins that are destined for the ER, the Golgi apparatus, lysosomes as well as those that are to be secreted from the cell are first delivered to the ER by ribosomes that associate with the membrane of the rough ER and synthesize the protein directly into the ER. Proteins delivered by this manner into the lumen of the ER undergo folding and modification in the ER. All proteins delivered to the ER, regardless of their final destination, have an N-terminal ER signal sequence of 15-30 amino acids.

Protein delivery into the endoplasmic reticulum

The N-terminal part of a protein is the first part of a nascent polypeptide that emerges from the ribosome (Figure 7.100). The sequence of amino acids in this region, if it is an ER signal, will be recognized and bound by a ribonucleoprotein complex called the Signal Recognition Particle (SRP). Binding of the SRP to the N-terminal signal sequence causes translation to pause. The SRP, in turn, is bound by an SRP receptor in the ER membrane (Movie 7.3 & Figure 7.101), effectively anchoring the ribosome to the membrane.

The location of SRP receptors near membrane channels in the ER positions the ribosome over a translocation channel. Once the ribosome is docked over the channel, the SRP releases the signal sequence, which is threaded through the channel, with its hydrophobic residues interacting with the hydrophobic interior of the membrane. Translation resumes at this point and the rest of the protein is delivered into the lumen of the ER as it is made. The ribosome remains associated with the ER membrane till translation is completed, at which point it dissociates. The signal sequence, which is no longer needed once the protein has been delivered, is cleaved off by a membrane associated signal peptidase, releasing the completed protein into the ER lumen.

While soluble proteins are delivered into the ER, integral membrane proteins do not pass all the way through, but, instead, are anchored in the membrane of the ER by hydrophobic stop transfer sequences.

Folding and modification

Proteins in the lumen of the ER are folded with the help of numerous chaperones resident in the endoplasmic reticulum. The environment within the ER lumen is also more oxidizing than the cytosol, and permits the formation of disulfide bonds to stabilize the folded proteins. Protein disulfide isomerase, an enzyme active in the ER lumen both helps to make disulfide bonds and removes bonds that were incorrectly made during the folding process. In addition, proteins in the ER undergo modifications such as glycosylation and addition of glycolipids. Multimeric proteins are also assembled from their subunits in the ER.

Proteins that have been correctly folded and modified are transported from the ER, in membrane vesicles, to their final destinations. Improperly folded proteins are recognized by a surveillance mechanism in the ER and are sent back to the cytoplasm to be degraded in proteasomes.

Information Processing: Translation

779

YouTube Lectures

by Kevin

HERE & HERE

780

Figure 7.81 - The standard genetic code

Wikipedia

Figure 7.80 - The central dogma in a bacterial cell

Wikipedia

781

Figure 7.82 - Coding in DNA, transcribed to RNA, translated to protein

782

Figure 7.83 - tRNA - 3D projection (left) and 2D projection (inset)

Wikipedia

783

Interactive 7.1 - Phenylalanyl-tRNA

PDB
Interactive 7.1 - Phenylalanyl-tRNA PDB

Figure 7.84 - Charging of a tRNA by aminoacyl tRNA synthetase

Wikipedia

784

Figure 7.85 - Codons in mRNA pair with anticodons on tRNA to bring the appropriate amino acid to the ribosome for polypeptide assembly

YouTube Lectures

by Kevin

HERE & HERE

785

Figure 7.86 - The A, P, and E sites in a ribosome

Image by Martha Baker

Figure 7.87 - Overview of elongation

Wikipedia

Interactive Learning

Module

HERE

786

Movie 7.1 - 30S ribosomal subunit

Wikipedia
Movie 7.1 - 30S ribosomal subunit Wikipedia

Table 7.1 - Location and function of rRNAs.

rRNA Name

Prokaryotes

Eukaryotes

Function

5S

Large Subunit

Large Subunit

tRNA binding?

5.8S

Large Subunit

Translocation?

16S

Small Subunit

mRNA alignment

18S

Large Subunit

mRNA alignment

23S

Large Subunit

Peptide bond formation

28S

Large Subunit

Peptide bond formation

787

Figure 7.89 - Conserved sequences adjacent to start codons for various bacterial genes

Image by Martha Baker

Figure 7.88 - Structure of 5S rRNA

788

Figure 7.90 - Base pairing between the Shine-Dalgarno sequence in the mRNA and the 16S rRNA

Image by Martha Baker

Movie 7.2 - Large ribosomal subunit

Wikipedia

Movie 7.2 - Large ribosomal subunit Wikipedia

789

YouTube Lectures

by Kevin

HERE & HERE

Figure 7.91 - Initiation - assembly of the ribosomal translation complex

Image by Martha Baker

790

Figure 7.92 - Kozak sequence plot showing relative abundance of bases surrounding the AUG (ATG) start codon of human genes

Figure 7.93 - EF-Tu (blue) bound to tRNA (red) and GTP (yellow)

791

Figure 7.94 - The process of elongation

Image by Martha Baker

792

Figure 7.95 - 50S ribosomal subunit. RNA in brown. Protein in blue. Peptidyl transferase site in red.

Wikipedia

Interactive Learning

Module

HERE

793

Figure 7.96 - The process of translation

Wikipedia

Movie 7.3 Translation of a protein secreted into the endoplasmic reticulum. Small subunit in yellow. Large subunit in green. tRNAs in blue.

Wikipedia
Movie 7.3 Translation of a protein secreted into the endoplasmic reticulum. Small subunit in yellow. Large subunit in green. tRNAs in blue. Wikipedia

794

Figure 7.97 - Another perspective of translation. The 3’ end of the mRNA is on the left and the ribosome is moving from right to left

795

Figure 7.98 - Action of chaperone to facilitate proper folding of a protein (orange)

Image by Aleia Kim

YouTube Lectures

by Kevin

HERE & HERE

796

Figure 7.99 - Rough (ribosome bound) and smooth endoplasmic reticulum

Wikipedia

797

Figure 7.100 - N-terminal signal sequence (green) emerging from the ribosome.

798

Figure 7.101 - Translation of a protein into the endoplasmic reticulum

Image by Aleia Kim

799

Graphic images in this book were products of the work of several talented students. Links to their Web pages are below

Click HERE for

Martha Baker's

Web Page

Click HERE for

Pehr Jacobson's

Web Page

Click HERE for

Aleia Kim's

Web Page

Click HERE for

Penelope Irving's

Web Page

Problem set related to this section HERE

Point by Point summary of this section HERE

To get a certificate for mastering this section of the book, click HERE

Kevin Ahern's free iTunes U Courses - Basic / Med School / Advanced

Biochemistry Free & Easy (our other book) HERE / Facebook Page

Kevin and Indira's Guide to Getting into Medical School - iTunes U Course / Book

To see Kevin Ahern's OSU ecampus courses - BB 350 / BB 450 / BB 451

To register for Kevin Ahern's OSU ecampus courses - BB 350 / BB 450 / BB 451

Biochemistry Free For All Facebook Page (please like us)

Kevin Ahern's Web Page / Facebook Page / Taralyn Tan's Web Page

Kevin Ahern's free downloads HERE

OSU's Biochemistry/Biophysics program HERE

OSU's College of Science HERE

Oregon State University HERE

Email Kevin Ahern / Indira Rajagopal / Taralyn Tan

801

Translation

To the tune of "Maria" (from "West Side Story")

Metabolic Melodies Website HERE

Translation
The most intricate thing I ever saw

From five prime to three prime, translation, translation
The final step that we know about the central dog-ma

Amino, carboxyl, translation, translation. . . .
Translation, translation, translation . .

Translation!

I just learned the steps of translation
And all the things they say
About tRNA
Are true

Translation!
To form peptide bonds in translation
The ribosomal cleft
Must bind to an E-F
tee-you!

Translation!
A-U-G binds the f-met's cargo
16S lines up Shine and Dalgarno
Translation
I'll never stop needing translation

The most intricate thing I ever saw
Translationnnnnnnnnnnnnnnnn

Recording by Tim Karplus

Lyrics by Kevin Ahern
Recording by Tim Karplus Lyrics by Kevin Ahern

802

Good Protein Synthesis

To the tune of “Good King Wenceslaus”

Metabolic Melodies Website HERE

Amino acids cannot join

By themselves together

They require ribosomes

To create the tether

All the protein chains get made

‘Cording to instruction

Carried by m-R-N-A

In peptide bond construction

Small subunit starts it all

With initiation

Pairing up two RNAs

At the docking station

Shine Dalgarno’s complement

In the 16 esses

Lines the A-U-G up so

Synthesis commences

Elongation happens in

Ribosomic insides

Where rRNA creates

Bonds for polypeptides

These depart the ribosome

Passing right straight through it

In the tiny channels there

Of the large subunit

Finally when the sequence of

One of the stop codons

Parks itself in the A site

Synthesis can’t go on

P-site RNA lets go

Of what it was holding

So the polypeptide can

Get on with its folding

Recording by David Simmons

Lyrics by Kevin Ahern

Recording by David Simmons Lyrics by Kevin Ahern

This page titled 4.8: Translation is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

4.9: Gene Expression

Source: BiochemFFA_7_7.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

The processes of transcription and translation described so far tell us what steps are involved in the copying of information from a gene (DNA) into RNA and the synthesis of a protein directed by the sequence of the transcript (Figure 7.102). These steps are required for gene expression, the process by which information in DNA directs the production of the proteins needed by the cell.

But what determines whether a gene is expressed at a given time? Cells do not, as we know, express all of their genes all of the time. Some genes are expressed in particular cell types but not others, while others may be expressed at specific stages of development. Cells must also be able alter their patterns of gene expression in response to internal and external cues, controlling the production of proteins as needed, to meet their changing needs. Regulating gene expression is, therefore, crucial. Given that there are multiple steps involved in gene expression, there are several different points at which the process could be regulated. Not surprisingly, many regulatory mechanisms are known, each acting at a different stage in the path from DNA to protein.

Regulation of Transcription

The first step in gene expression is transcription, so regulation of transcription is an obvious way to affect whether a gene is expressed and to what extent.

What are the molecular switches that turn transcription on or off? Although there are additional factors that affect transcription, such as the accessibility of a gene to the transcriptional machinery, the basic mechanism by which transcription is regulated depends on highly specific interactions between transcription regulating proteins and regulatory sequences on DNA.

What are these regulatory sequences and what proteins bind them? In addition to the promoter sequences required for transcription initiation, genes have additional cis regulatory sequences (sequences of DNA on the same DNA molecule as the gene) that control when a gene is transcribed. Regulatory sequences are bound tightly and specifically by transcriptional regulators, proteins that can recognize DNA sequences and bind to them. The binding of such proteins to the DNA can regulate transcription by preventing or increasing transcription from a particular promoter.

Transcriptional regulation in prokaryotes

Let us first consider some examples from prokaryotes. In bacteria, genes are often clustered in groups, such that genes that need to be expressed at the same time are next to each other and all of them are controlled as a single unit by the same promoter. Groups of genes that are coordinately regulated by a single promoter are referred to as operons. The entire set of genes in an operon can be controlled through the action of DNA binding proteins that act as either repressors (preventing transcription of the genes) or activators (increasing transcription of the genes). The binding of these proteins to their DNA targets is allosterically controlled by the binding of specific small molecules that signal the state of the cell.

Induction of the lac operon

The lac operon is one such group of coordinately regulated genes that encode proteins needed for the uptake and breakdown of the sugar lactose. E.coli cells preferentially use glucose for their energy needs, but if glucose is unavailable, and lactose is present, the bacteria will take up lactose and break it down for energy. Since the proteins for taking up and breaking down lactose are only needed when glucose is absent and lactose is available, the bacterial cells need a way to express the genes of the lac operon only under those conditions. The default state of the lac operon is OFF.

Removing a repressor

Transcription of the lac cluster of genes is primarily controlled by a repressor protein that binds to a region of the DNA just downstream of the -10 sequence of the lac promoter (Figure 7.104). Recall that the promoter is where the RNA polymerase must bind to begin transcription. The location on the DNA where the lac repressor is bound is called the operator (Figure 7.105). When the repressor is bound at this position, it physically blocks the RNA polymerase from transcribing the genes, just as a vehicle blocking your driveway would prevent you from pulling out. Obviously, if you want to leave, the vehicle that is blocking your path

must be removed. Likewise, in order for transcription to occur, the repressor must be removed from the operator to clear the path for RNA polymerase (Figure 7.106).

How is the repressor removed? When the sugar lactose is present, a small amount of it is taken up by the cells and converted to an isomeric form, allolactose (Figure 7.107). Allolactose binds to the repressor, changing its conformation so that it no longer binds to the operator. When the repressor is no longer bound to the operator, the "road-block" in front of the RNA polymerase is removed, permitting the transcription of the genes of the lac operon

What makes this an especially effective control system is that the genes of the lac operon encode proteins that enable the break down of lactose. Turning on these genes requires lactose to be present. Once the lactose has been broken down, the lac repressor binds to the operator once more and the lac genes are no longer expressed. This allows the genes to be expressed only when they are needed.

Recruiting RNA polymerase

But how do glucose levels affect the expression of the lac genes? We noted earlier that if glucose was present, lactose would not be used. A second level of control is exerted by a protein called Catabolite Activator Protein (CAP - Figure 7.108)). CAP (also sometimes called CBP or cAMP binding protein) binds to a site adjacent to the promoter and is necessary to recruit RNA polymerase to bind the lac promoter.

cAMP binding

CAP binds to its site only when glucose levels are low. Low glucose levels are linked to the activation of an enzyme, adenylate cyclase, that makes the molecule cyclic AMP (cAMP). The binding of cAMP to the CAP causes a conformational change in CAP that allows it to bind to the CAP-binding site. When CAP is bound at this site, it is able to recruit RNA polymerase to bind at the promoter, and begin transcription.

The combination of CAP binding and the lac repressor dissociating from the operator when lactose levels are high ensures transcription of the lac operon just when it is most needed. The binding of CAP may be thought of as a green light for the RNA polymerase, while the removal of lac repressor is like the lifting of a barricade in front of it. When both conditions are met, the RNA polymerase transcribes the downstream genes.

Control of the trp operon by repression

The lac operon we have just described is a set of genes that are expressed only under the specific conditions of glucose depletion and lactose availability. Other genes may be expressed unless a particular condition is met. For these genes, the default state is ON.

An example of this is the trp operon, which encodes enzymes necessary for the synthesis of the amino acid tryptophan. These genes are constitutively expressed (always on), except when tryptophan is available from the cell's surroundings, making its synthesis unnecessary. Under conditions where tryptophan is abundant in the environment, the trp genes can be turned off. This is achieved by a repressor protein that will bind to the operator only in the presence of tryptophan (Figure 7.110). Binding of tryptophan to the repressor causes binding of the repressor to the operator. Because it acts together with the repressor to turn off the trp genes, tryptophan is called a co-repressor.

Attenuation

Another mechanism that regulates the expression of the trp operon is attenuation. Attenuation is a process by which the expression of an operon is controlled by termination of transcription before the first gene of the operon (Figure 7.111).

In the trp operon, this functions as follows: Transcription begins some distance upstream of the first gene in the operon, producing what is termed a 5’ leader sequence. This leader sequence contains an intrinsic terminator that can form a hairpin structure that stops transcription when high levels of tryptophan are available to the cells. It can also form a different structure that permits continued transcription of the genes in the operon when tryptophan levels are low. How does the level of tryptophan influence which of these two structures are formed?

Recall that the 5’ end of the RNA is the first part of the transcript to be made and that in bacteria translation is linked to transcription, so the 5’ end of the RNA begins to be translated before the entire transcript is made. It turns out that the 5’ leader sequence of the trp operon mRNA encodes a short peptide that contains two tryptophan codons. If there is plenty of tryptophan

available, the leader sequence will be easily translated. Under these conditions, the leader sequence is able to form the termination hairpin, preventing the transcription of the downstream trp genes.

If, however, levels of tryptophan are low, then the ribosome stalls as it attempts to translate the leader sequence. Under these conditions, the leader sequence adopts a different conformation that permits continued transcription of the genes of the trp operon.

Riboswitches

Similar in concept to the attenuation of the trp operon described above, but not dependent on translation, is a control mechanism called a riboswitch (Figure 7.113). Riboswitches are typically found in the 5'UTR of messenger RNAs (i.e., they are part of the sequence of the RNA). These sequences can control transcription of the downstream genes based on the conformation they adopt. One conformation allows continued transcription, while the other terminates it. So, what determines which conformation they adopt?

Features

Riboswitches have two characteristic features that are important for their function. One is a region of the sequence called an aptamer, which folds into a three-dimensional shape that can bind a small effector molecule. The other is an adjacent region of the RNA, called the expression platform, that can fold into different conformations depending on whether or not the aptamer is bound to the effector.

An example of a riboswitch found in bacteria is the guanine riboswitch, which controls the expression of genes required for purine biosynthesis. The aptamer region of this riboswitch binds to the effector, guanine, when levels of the base are high. The binding of the guanine triggers a change in the folding of the downstream expression platform, causing it to adopt a conformation that terminates transcription of the genes needed for the synthesis of guanine. In the absence of guanine, the expression platform assumes a different conformation that allows transcription of the purine biosynthesis genes. Thus, levels of guanine can be sensed and the genes needed for its synthesis can be expressed as needed.

Regulation of transcription in eukaryotes

Transcription in eukaryotes is also regulated by the binding of proteins to specific DNA sequences, but with some differences from the simple schemes outlined above.

For most eukaryotic genes, general transcription factors and RNA polymerase (i.e., the transcription initiation complex) are necessary but not sufficient for high levels of transcription. Promoter-proximal DNA sequences like the CAAT box and GC box bind proteins that interact with the transcription initiation complex, influencing its formation (Figure 7.114).

Distant regulatory sequences

Additional regulatory sequences called enhancers and the proteins that bind to them are needed to achieve high levels of transcription. Enhancers are short DNA sequences that regulate the transcription of genes, but may be located at a distance from the gene they control (although they are on the same DNA molecule as the gene). Often enhancers are many kilobases away on the DNA, either upstream or downstream of the gene. As the name suggests, enhancers can enhance (increase) transcription of a particular gene. How can a DNA sequence far from the gene being transcribed affect the level of transcription?

Transcriptional activators

Enhancers work by binding proteins (transcriptional activators) that can, in turn, interact with the proteins bound at the promoter. The enhancer region of the DNA, with its associated transcriptional activator(s) can come in contact with the transcription initiation complex that is bound at a distant site by looping of the DNA (Figure 7.115). This allows the protein bound at the enhancer to make contact with the proteins in the basal transcription complex. The interaction of the activator with the transcription initiation complex may be direct, or it may be through a “middle-man”, a protein complex called mediator.

One effect of this interaction is to assist in recruiting proteins necessary for transcription, like the general transcription factors and RNA polymerase to the promoter, increasing the frequency and efficiency of formation of the transcription initiation complex. There is also evidence that at some promoters, following assembly of the transcription initiation complex, the RNA polymerase remains stalled at the promoter. In such cases, the interaction with the transcription initiation complex of an activator bound to an enhancer could play a role in facilitating the transition of the RNA polymerase to the elongation phase of transcription.

Chromatin remodeling proteins

Another mechanism by which activators bound at the enhancer can affect transcription is by recruiting to the promoter proteins that can modify the structure of that region of the chromosome. In eukaryotes, DNA is packaged with proteins to form chromatin. When the DNA is tightly associated with these proteins, it is difficult to access for transcription. So proteins that can make the DNA more accessible to the transcription machinery can also play a role in the extent to which transcription occurs.

Silencers

In addition to enhancers, there are also negative regulatory sequences called silencers. Such regulatory sequences bind to transcriptional repressor proteins. Like the transcriptional activators, these repressors work by interacting with the transcription initiation complex. In the case of repressors, the effect they have on the transcription initiation complex is to reduce transcription.

DNA binding proteins

Transcriptional activators and repressors are modular proteins- they have a part that binds DNA and a part that activates or represses transcription by interacting with the transcription initiation complex (Figure 7.118). The DNA binding domain is the part of the protein that confers specificity for determining which gene(s) will be activated or repressed. The activation domain is the part of the protein that stimulates or represses transcription. The DNA binding domains of transcriptional activators form characteristic structures that recognize their target DNA sequences by making contacts with bases, usually in the major groove of the DNA helix. It is possible to engineer hybrid transcription factors that combine the DNA binding domain of one activator with the activation domain of another. Such proteins retain the specificity dictated by the DNA binding domain. Truncated transcription factors can also be generated that have their DNA binding domain but lack the activation domain. Such transcription factors can be useful tools in studying transcriptional regulation because their DNA binding domains can compete with the endogenous transcription factors for regulatory binding sites without increasing transcription from the target promoters.

Multiple factors

The description above may suggest that each gene in eukaryotes is controlled by the binding of a single transcriptional activator or repressor to a particular enhancer or silencer site. However, it turns out that the transcription of any given gene may be simultaneously regulated by a combination of proteins, both activators and repressors, bound at multiple regulatory sites on the DNA, all of which interact with the transcription initiation complex. The combinatorial nature of such regulation provides great versatility, with different combinations of regulatory elements and proteins working together in response to a wide variety of conditions and signals.

The mechanisms described so far have focused on the sequence elements in DNA that regulate transcription through the activator and repressor proteins bound to them. Following transcription, alternative splicing (see HERE) and editing of the transcripts can also modify the proteins that are produced by the cell. We will now examine some of the other ways in which gene expression is modulated in cells.

First, we will consider some so-called epigenetic mechanisms that affect gene expression. The term epigenetics derives from epi (above, or on top of) and genetic (of genes) and refers to the fact that these mechanisms act in addition to, or overlaid on, the information in the gene sequences. Two such epigenetic mechanisms are the covalent modifications of histones in chromatin and the methylation of DNA sequences.

Histone modification

As noted earlier, transcription in eukaryotes is complicated by the fact that the DNA is packaged with histones to make chromatin. This means that for a gene to be transcribed, the relevant regions of the chromatin must be opened up to allow access to the RNA polymerase and transcription factors. This provides another potential point of control of gene expression. Chromatin remodeling factors, mentioned earlier, assist in reorganizing the nucleosome structure at regions that need to be made accessible.

But what determines that a given region of the chromatin will be acted upon by the remodeling complexes? Transcriptional activator proteins bound at enhancers, sometimes work by recruiting histone modifying enzymes to the promoter region. An example of such a modifying enzyme is histone acetyl transferase (HAT) that works to acetylate specific amino acid residues in the tails of the histones forming the nucleosome core (Figures 7.119 & 7.120). Acetylation of histones is thought to be responsible for loosening the interaction between histones and the DNA in nucleosomes and helps to make the DNA more readily accessible for

transcription. The opposite effect may be achieved if the enzymes recruited are histone deacetylases (HDAC) which remove acetyl groups from the tails of the histones in the nucleosome, and lead to tighter packing of the chromatin.

Writers, readers and erasers

In addition to the histone acetyl transferases and the deacetylases, other enzymes may add or remove methyl groups, phosphate groups, and other chemical moieties to specific amino acid side chains on the histone tails. The patterns of these covalent modifications, sometimes called the histone code, are established by the so-called "writers", or enzymes, such as histone methyltransferases, that add the chemical groups on to the histone tails. Yet other enzymes, like the histone demethylases, may act as "erasers," removing the chemical groups added by the "writers." The histone code is interpreted by "readers," proteins that bind to specific combinations of the modifications and assist in either silencing the expression of genes in the vicinity or making the region more transcriptionally active.

DNA methylation

Gene expression can also be regulated by methylation of the other component of chromatin - DNA. Enzymes called DNA methyltransferases (DNMTs) catalyze the covalent addition of a methyl group to C5 of cytosines in DNA. Patterns of cytosine methylation vary in different organisms, with methylation concentrated in some parts of the genome in some groups and scattered throughout the genome in others. In vertebrates, the cytosines that are methylated are generally next to a guanine (the CG dinucleotide is commonly abbreviated as CpG). Methylation of DNA seems to correlate with gene silencing while demethylation is associated with increased transcription (Figure 7.121).

How does methylation of the DNA at CpG sites regulate gene expression? Although the extent of DNA methylation near promoters has been observed to correlate with gene silencing, it is not clear how exactly methylation brings about this effect. It has been suggested that methylation could block the binding of proteins necessary for transcription. Methylation at enhancer sites might also prevent the binding of transcriptional activators to them.

Another interesting observation is that certain proteins that bind to methylated CpG sites also seem to interact with histone deacetylases. As noted above, histones deacetylases remove acetyl groups from histones, and promote tighter packing of chromatin and transcriptional silencing. Thus, methylation on DNA likely works in combination with histone modification to affect gene expression.

Regulatory RNAs

One of the most unexpected discoveries in the past few decades has been the role that RNAs play in regulating gene expression. The classic view that RNA either encoded proteins (mRNA) or assisted in their synthesis (rRNA and tRNA) is now known to be a vast underestimate of the various ways in which RNAs function in gene expression. It is now clear that regulatory RNAs have widespread and significant effects on gene expression, a realization that has revolutionized our understanding of gene regulation.

What are some of the ways in which regulatory RNAs function to modulate the expression of genes?

Small regulatory RNAs

MicroRNAs (miRNAs) and Short Interfering RNAs (siRNAs) are small, non-coding RNAs that act at the post-transcriptional level to regulate gene expression (Figure 7.123 & 7.124). These RNAs appear to silence genes by base-pairing with target mRNAs and marking them for degradation, or by blocking their translation. The functional forms of both miRNAs and siRNAs are from 20-30 nucleotides long and are derived by processing from longer primary transcripts. Mature miRNAs and siRNAs work in association with a class of proteins called Argonaute proteins to form a gene silencing complex.

MicroRNAs are transcribed from specific genes by RNA polymerase II. The primary transcript, known as a pri-miRNA folds on itself to form double-stranded hairpin structures that are cleaved by an RNase in the nucleus called Drosha. The products of Drosha cleavage, double-stranded RNAs of roughly 60-70 nucleotides known as pre-miRNAs, are exported to the cytoplasm, where they are further processed into the small 20-30 nucleotide lengths of mature double-stranded miRNAs by an enzyme known as Dicer. The RNA duplexes of miRNAs are not perfectly matched, and have loops and mismatches (Figure 7.124).

siRNAs also derive from double-stranded RNAs, but these may arise from either endogenous or exogenous sources (such as viruses). These double-stranded RNAs are processed in the cytoplasm by the same enzyme, Dicer, that generates the mature miRNAs, to produce the small, 20-30 nucleotide double-stranded RNAs.

In contrast to miRNAs, the mature siRNAs are perfectly base-paired along their lengths.

RISC assembly

Both miRNAs and siRNAs then are assembled with Argonaute proteins to form a silencing complex called RISC (RNA-induced silencing complex). Recall that both miRNAs and siRNAs are, at this point double-stranded. One strand of the RNA is referred to as the guide RNA, while the other is called the passenger RNA.

During the process of loading the RNA onto the Argonaute protein, the guide strand of the RNA remains associated with the protein, while the passenger strand is removed. The guide RNA associated with the Argonaute protein is the functional gene silencing complex (Figure 7.125).

Sequence specific base-pairing of the guide RNA with an mRNA leads to either the degradation of the mRNA by the Argonaute protein (in the case of the siRNAs) or in suppression of translation of the mRNA (for miRNAs). The extent to which these processes play a role in regulating gene expression is impressive. The expression of at least a third of all human genes has already been shown to be modulated by miRNAs, demonstrating clearly that these RNAs play a major role in gene regulation.

Long noncoding RNAs

Long noncoding RNAs (lncRNAs) are RNAs of greater than 200 nucleotides that do not code for proteins. Some of these RNAs are derived from intron sequences, while others, transcribed from intergenic regions form a subset of lncRNAs called lincRNAs (long intergenic noncoding RNAs). Yet other lncRNAs are produced as antisense transcripts of coding genes. An astounding 30,000 transcripts in humans are thought to be lncRNAs, but little is known of their function. From the few lncRNAs that have been intensively studied, it is evident that they do not all function in the same way. However, they appear to affect gene expression in a variety of ways including modification of chromatin structure, regulation of splicing, or serving as structural scaffolds for the assembly of nucleoprotein complexes. Additional mechanisms will doubtless be uncovered as these fascinating RNAs are investigated in years to come.

Regulation of translation

The synthesis of proteins is dependent on the availability of the mRNAs encoding them. If an mRNA is blocked at its 5' end, it cannot be translated. The rate of degradation of an mRNA will influence how long it is around to direct the synthesis of the protein it codes for. Gene expression can also, therefore, be regulated by mechanisms that alter the rate of mRNA degradation. Regulation of translation is used to control the production of many proteins. Two examples, ferritin and the transferrin receptor, are important for iron storage and transport in cells. Ferritin is an iron-binding protein that sequesters iron atoms in cells to keep them from reacting. When iron levels are high, there is a need for more ferritin than when iron levels are low. How are ferritin levels regulated? The 5'UTR of the ferritin mRNA contains a 28-nucleotide sequence called the Iron Response Element, or IRE (Figure 7.127). When iron levels are low, the IRE is bound by a protein. The presence of the IRE-binding protein at the 5'UTR blocks translation of the ferritin mRNA. However, if iron levels are high, the iron binds to the IRE-binding protein, which undergoes a conformational change and dissociates from the IRE. This frees up the 5' end of the ferritin mRNA for ribosome assembly and translation, producing more ferritin.

The other protein involved in iron transport, the transferrin receptor, is required for uptake of iron into cells, when intracellular iron levels are low. In the case of the transferrin receptor, it is when iron levels are low that more of it is needed. When iron levels are high, there is no need to make more transferrin receptor. The mRNA encoding the transferrin receptor also has IRE sequences, but in this case, the IRE is situated in the 3'UTR of the transcript (Figure 7.128). The IRE is, as in the case of ferritin, bound by the IRE-binding protein. When iron levels in the cell are high, the iron binds the IRE-binding protein, which dissociates from the IRE. This leaves the 3'UTR susceptible to attack by RNases, leading to degradation of the transferrin receptor mRNA. At times when iron levels are low, the IRE-binding protein remains bound to the 3' UTR of the mRNA, stabilizing it and permitting more transferrin receptor to be made by translation.

Gene expression is controlled at many steps

As can be seen from the examples in this section, regulation of gene expression in eukaryotic cells is a function of multiple mechanisms that act at different stages in the flow of information from DNA to protein, responding to the internal state of the cell as well as external conditions and signals.

Information Processing: Gene Expression

803

YouTube Lectures

by Kevin

HERE & HERE

804

Figure 7.102 - Multiple levels of control of gene expression

Wikipedia

805

Figure 7.103 - Prokaryotic genes organized in an operon

Wikipedia

Figure 7.104 - Protein binding sites in the lac regulatory region

Image by Martha Baker

Interactive Learning

Module

HERE

806

Figure 7.105 - Lac operon structure and products

Image by Martha Baker

Figure 7.106 - Lac operon in the absence (middle) and presence (bottom) of inducer

Image by Martha Baker

807

Figure 7.107 - Allolactose (top) and lactose (bottom)

Figure 7.108 - CAP (blue) bound to the DNA adjacent to the lac promoter (orange). cAMP shown in pink.

Wikipedia

Figure 7.109 - Lac operon in the presence (top) and absence (bottom) of glucose

Image by Martha Baker

808

Figure 7.110 - Structure and regulation of the trp operon

Wikipedia

YouTube Lectures

by Kevin

HERE & HERE

809

Figure 7.111 - Attenuation in regulation of the trp operon

Wikipedia

Figure 7.112 - Sequence of the leader region of the trp operon

XX AUG AAA GCA AUU UUC GUA CUG AAA GGU UGG UGG CGC ACU UCC UGA -XX

MET LYS ALA ILE PHE VAL LEU LYS GLY TRP TRP ARG THR SER STOP

810

Figure 7.113 - Riboswitch features

811

Figure 7.114 - Regulatory sequences for a eukaryotic gene

Wikipedia

812

Figure 7.115 - DNA looping allows contact between activator bound at a distant enhancer and the basal transcription complex

Image by Martha Baker

813

Figure 7.116 - Transcription factors in regulation of eukaryotic transcription

Wikipedia

YouTube Lectures

by Kevin

HERE & HERE

814

Figure 7.117 - Binding of c-myc protein to its target DNA sequence

Wikipedia

Figure 7.118 Activators bound at multiple sites can regulate transcription from a given promoter

OpenStax

815

Figure 7.119 - Transcriptional activation (right) and deactivation (left) by histone modification

Wikipedia

816

Figure 7.120 - Chromatin configuration affects transcription

Wikipedia

Interactive Learning

Module

HERE

817

Figure 7.121 - Inactivation of transcription by CpG methylation

Image by Indira Rajagopal

YouTube Lectures

by Kevin

HERE & HERE

818

Figure 7.122 - Epigenetic changes through histone and DNA modification

819

Figure 7.123 - miRNAs function in the regulation of gene expression

Wikipedia

Figure 7.124 Pre-miRNA hairpin structures with the mature guide miRNAs shown in red

Wikipedia

820

Figure 7.125 - Gene silencing by siRNA

Image by Pehr Jacobson

821

Figure 7.126 - Processed siRNA duplex with perfect base-pairing, 5’ phosphates and two bases overhanging at each 3’ end

822

Figure 7.127 -Regulation of ferritin mRNA translation

Image by Aleia Kim

YouTube Lectures

by Kevin

HERE & HERE

823

Figure 7.128 -Regulation of transferrin receptor mRNA translation

Image by Aleia Kim

Graphic images in this book were products of the work of several talented students. Links to their Web pages are below

Click HERE for

Martha Baker’s

Web Page

Click HERE for

Pehr Jacobson’s

Web Page

Click HERE for

Aleia Kim’s

Web Page

Click HERE for

Penelope Irving’s

Web Page

Problem set related to this section HERE

Point by Point summary of this section HERE

To get a certificate for mastering this section of the book, click HERE

Kevin Ahern’s free iTunes U Courses - Basic / Med School / Advanced

Biochemistry Free & Easy (our other book) HERE / Facebook Page

Kevin and Indira's Guide to Getting into Medical School - iTunes U Course / Book

To see Kevin Ahern's OSU ecampus courses - BB 350 / BB 450 / BB 451

To register for Kevin Ahern's OSU ecampus courses - BB 350 / BB 450 / BB 451

Biochemistry Free For All Facebook Page (please like us)

Kevin Ahern's Web Page / Facebook Page / Taralyn Tan's Web Page

Kevin Ahern's free downloads HERE

OSU's Biochemistry/Biophysics program HERE

OSU's College of Science HERE

Oregon State University HERE

Email Kevin Ahern / Indira Rajagopal / Taralyn Tan

God Bless These Complexes

To the tune of "God Bless America"

Metabolic Melodies Website HERE

All information in

Cells' DNA

Just increases

With pieces

Mixed and matched in the mRNAs

Linking exons

All together

Using snurps in

Complex-ES

God bless the spliceosomes

And trans-crip-tomes

(slow and loud) God bless the spliceosomes

And my ge-nome

Your blueprint info is

In DNA

Since you need it

Proofread it

Or you'll mutate the mRNA

You can translate

All the codons

With the cells' gen-

et-ic code

God bless the ribosomes

They translate code

(slow and loud) God bless the ribosomes

And proteomes

Recording by David Simmons

Lyrics by Kevin Ahern

Recording by David Simmons Lyrics by Kevin Ahern

The Book of Life

To the tune of “The Look of Love”

Metabolic Melodies Website HERE

The book of life - the stuff of dreams

Is everywhere, it seems

The book of life, is biochemistry and

Its words fill every day

Just what it says is written in the DNA

I just want to get to know it

How the info’s coded

What are all the secrets?

Ribosomes can read it

Goodness knows it’s needed

And so its alphabet’s

In codon forms

For ribosome bookworms

They read it right

A protein’s function to its sequence corresponds

It’s not just randomly created peptide bonds

What a marvel of creation, how they do translation

Of m-R-N-A chains,

Using bits of glycine

Proline and some lysine

Translate the code

Instrumental

I just marvel at the knowledge

That I got in college

To learn all the secrets

Double helix spaces

Complementary bases

Pyrimidines

Paired to purines

The book of life

Recording by Carol Adriane Smith

Lyrics by Kevin Ahern
Recording by Carol Adriane Smith Lyrics by Kevin Ahern

This page titled 4.9: Gene Expression is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

CHAPTER OVERVIEW

5: Structure and Function- Carbohydrates and Lipids

5.1: Structure and Function- Carbohydrates

5.2: Structure and Function - Lipids and Their Transport

5.2.1: 5.2.1 Optional- other interesting lipids

5.2.1.1: Bonus- other interesting lipids

5.3: Basic Concepts in Membranes

5.4: Transport Across Membranes

5.4.1: Optional deep dive in to membrane transport

5.5: Other Considerations in Membranes

5: Structure and Function- Carbohydrates and Lipids is shared under a not declared license and was authored, remixed, and/or curated by LibreTexts.

5.1: Structure and Function- Carbohydrates



Figure 2.171 - Repeating unit of amylose

Endogenous glycation, on the other hand, arises with a frequency that is proportional to the concentration of free sugar in the body. These occur most frequently with fructose, galactose, and glucose in that decreasing order and are detected in the bloodstream. Both proteins and lipids can be glycated and the accumulation of endogenous advanced glycation endproducts (AGEs) is associated with Type 2 diabetes, as well as in increases in cardiovascular disease (damage to endothelium, cartilage, and fibrinogen), peripheral neuropathy (attack of myelin sheath), and deafness (loss of myelin sheath).

The formation of AGEs increases oxidative stress, but is also thought to be exacerbated by it. Increased oxidative stress, in turn causes additional harm. Damage to collagen in blood cells causes them to stiffen and weaken and is a factor in hardening of the arteries and formation of aneurysms, respectively. One indicator of diabetes is increased glycation of hemoglobin in red blood cells, since circulating sugar concentration are high in the blood of diabetics. Hemoglobin glycation is measured in testing for blood glucose control in diabetic patients.

Homopolymer	Monomeric Unit
Glycogen	Glucose
Cellulose	Glucose
Amylose	Glucose
Callose	Glucose
Chitin	N-acetylglucosamine
Xylan	Xylose
Mannan	Mannose
Chrysolaminarin	Glucose

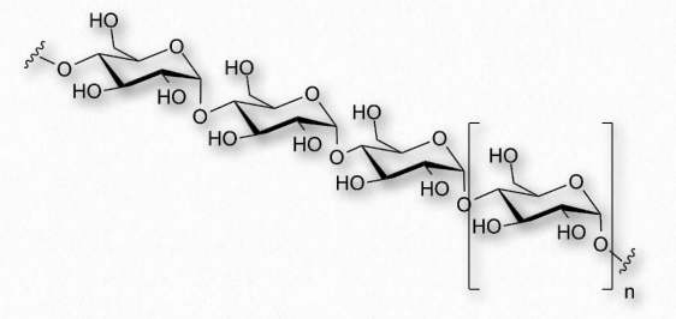


Figure 2.189). Along with the proteoglycan called lubricin, hyaluronic acid turns water into lubricating material. Hyaluronic acid is present as a coat around each cell of articular cartilage and forms complexes with proteoglycans that absorb water, giving resilience (resistance to compression) to cartilage. Aging causes a decrease in size of hyaluronans, but an increase in concentration.

Function in skin

Hyaluronic acid is a major component of skin and has functions in tissue repair. With exposure to excess UVB radiation, cells in the dermis produce less hyaluronan and increase its degradation.

For some cancers the plasma level of hyaluronic acid correlates with malignancy. Hyaluronic acid levels have been used as a marker for prostate and breast cancer and to follow disease progression. The compound can to used to induce healing after cataract surgery. Hyaluronic acid is also abundant in the granulation tissue matrix that replaces a fibrin clot during the healing of wounds. In wound healing, it is thought that large polymers of hyaluronic acid appear early and they physically make room for white blood cells to mediate an immune response.

Breakdown

Breakdown of hyaluronic acid is catalyzed by enzymes known as hyaluronidases. Humans have seven types of such enzymes, some of which act as tumor suppressors. Smaller hyaluronan fragments can induce inflammatory response in macrophages and dendritic cells after tissue damage. They can also perform proangiogenic functions.

Proteoglycans

Glycosaminoglycans are commonly found attached to proteins and these are referred to as proteoglycans. Linkage between the protein and the glycosaminoglycan is made through a serine side-chain. Proteoglycans are made by glycosylation of target proteins in the Golgi apparatus.

This page titled 5.1: Structure and Function- Carbohydrates is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **2.7: Structure and Function- Carbohydrates** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

5.2: Structure and Function - Lipids and Their Transport

Source: BiochemFFA_2_7.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy



Lipids are a diverse group of molecules that all share the characteristic that at least a portion of them is hydrophobic. Lipids play many roles in cells, including serving as energy storage (fats/oils), constituents of membranes (glycerophospholipids, sphingolipids, cholesterol), hormones (steroids), vitamins (fat soluble), oxygen/ electron carriers (heme), among others. For lipids that are very hydrophobic, such as fats/ oils, movement and storage in the aqueous environment of the body requires special structures. Other, amphipathic lipids, such as glycerophospholipids and sphingolipids spontaneously organize themselves into lipid bilayers when placed in water. Interestingly, major parts of many lipids can be derived from acetyl-CoA.

Fatty acids

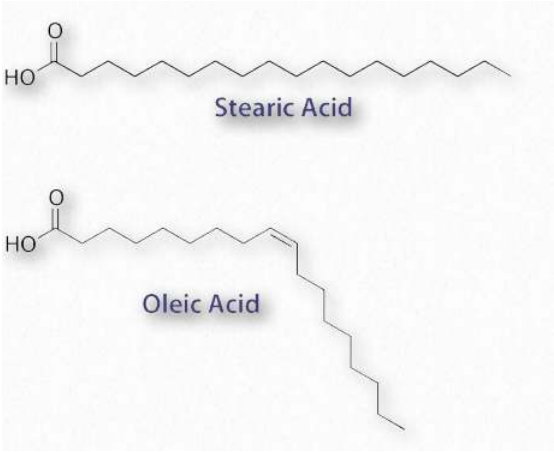


Figure 2.190 - Saturated fatty acid (stearic acid) and unsaturated fatty acid (oleic acid)

The most ubiquitous lipids in cells are the fatty acids. Found in fats, glycerophospholipids, sphingolipids and serving as as membrane anchors for proteins and other biomolecules, fatty acids are important for energy storage, membrane structure, and as precursors of most classes of lipids. Fatty acids, as can be seen from Figure 2.190 are characterized by a polar head group and a long hydrocarbon tail. Fatty acids with hydrocarbon tails that lack any double bonds are described as saturated, while those with one or more double bonds in their tails are known as unsaturated fatty acids. The effect of double bonds on the fatty acid tail is to introduce a kink, or bend, in the tail, as shown for oleic acid.

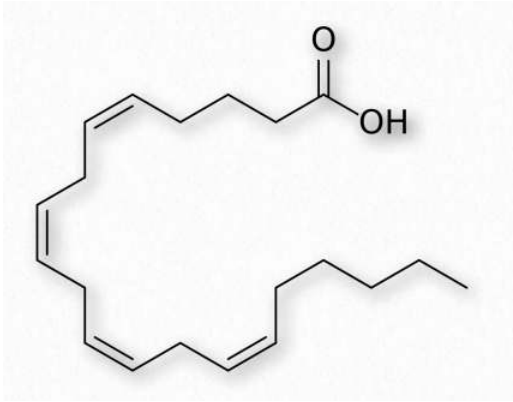


Figure 2.191 - Arachidonic acid - A polyunsaturated fatty acid Wikipedia

Stearic acid, a saturated fatty acid, by contrast has a straight hydrocarbon tail. Figures 2.190-2.194 show the most common saturated and unsaturated fatty acids. Fatty acids with unsaturated tails have a lower melting temperature than those with saturated tails of the same length. Shorter tails also decrease melting temperature. These properties carry over to the fats/oils containing them.

Common name	Chemical structure	
Caprylic acid	$CH_3(CH_2)_6COOH$	8
Capric acid	$CH_3(CH_2)_8COOH$	10
Lauric acid	$CH_3(CH_2)_{10}COOH$	12
Myristic acid	$CH_3(CH_2)_{12}COOH$	14
Palmitic acid	$CH_3(CH_2)_{14}COOH$	16
Stearic acid	$CH_3(CH_2)_{16}COOH$	18
Arachidic acid	$CH_3(CH_2)_{18}COOH$	20
Behenic acid	$CH_3(CH_2)_{20}COOH$	22
Lignoceric acid	$CH_3(CH_2)_{22}COOH$	24
Cerotic acid	$CH_3(CH_2)_{24}COOH$	26

Figure 2.192 - Saturated fatty acids. Number of carbons in right column Wikipedia

Fatty acids with more than one double bond are called polyunsaturated. Plants are excellent sources of unsaturated and polyunsaturated fatty acids. The position of the double bond(s) in fatty acids has important considerations both for their synthesis and for their actions in the body. Biochemically, the double bonds found in fatty acids are predominantly in the cis configuration. So-called trans fats arise as a chemical by-product of partial hydrogenation of vegetable oil.

Name	Double Bond Info	
Myristoleic acid	*cis*-Δ^9	14:1
Palmitoleic acid	*cis*-Δ^9	16:1
Sapienic acid	*cis*-Δ^6	16:1
Oleic acid	*cis*-Δ^9	18:1
Elaidic acid	*trans*-Δ^9	18:1
Vaccenic acid	*trans*-Δ^{11}	18:1
Linoleic acid	*cis,cis*-Δ^9,Δ^{12}	18:2
Linoelaidic acid	*trans,trans*-Δ^9,Δ^{12}	18:2
α-Linolenic acid	*cis,cis,cis*-$\Delta^9,\Delta^{12},\Delta^{15}$	18:3
Arachidonic acid	*cis,cis,cis,cis*-$\Delta^5\Delta^8,\Delta^{11},\Delta^{14}$	20:4
Eicosapentaenoic acid	*cis,cis,cis,cis,cis*-$\Delta^5,\Delta^8,\Delta^{11},\Delta^{14},\Delta^{17}$	20:5
Erucic acid	*cis*-Δ^{13}	22:1
Docosahexaenoic acid	*cis,cis,cis,cis,cis,cis*-$\Delta^4,\Delta^7,\Delta^{10},\Delta^{13},\Delta^{16},\Delta^{19}$	22:6

Figure 2.193 - Unsaturated fatty acids. Right column Indicates number of carbons and double bonds Wikipedia

In humans, consumption of trans fat raises low density lipoprotein (LDL) levels and lowers high density lipoprotein (HDL) levels. Each is thought to contribute to the risk of developing coronary artery disease. The most Figure 2.194 - Fatty acid models. Carboxyl end labeled in red Wikipedia common fatty acids in our body include palmitate, stearate, oleate, linolenate, linoleate, and arachidonate. Two notable shorter fatty acids are nonanoic (9 carbons) and decanoic acid (10 carbons), both of which appear to have anti-seizure effects. Decanoic acid directly inhibits excitatory neurotransmission in the brain and may contribute to the anticonvulsant effect of the ketogenic diet.

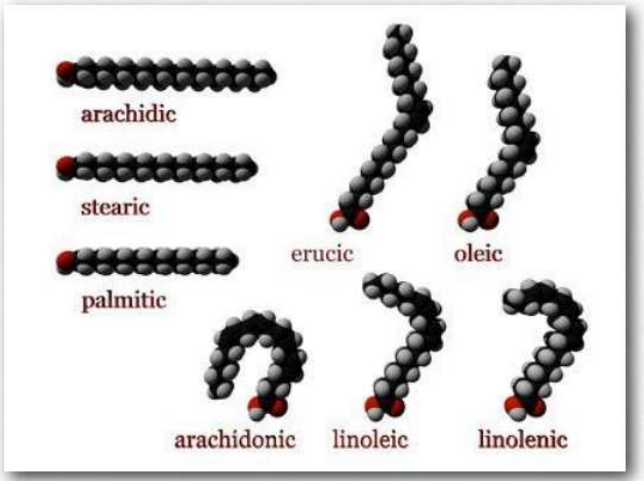


Figure 2.194 - Fatty acid models. Carboxyl end labeled in red Wikipedia

Numbering

Figure 2.195 shows two different systems for locating double bonds in a fatty acid. The ω system counts carbons starting with the methyl end (shown in red) while the Δ system counts from the carboxyl end (shown in blue). For example, an ω-3 (omega 3) fatty acid would have a double bond at the third carbon from the methyl end. In the Δ system, a fatty acid that has a cis double bond at carbon 6, counting from the carboxyl end, would be written as cis-Δ6.

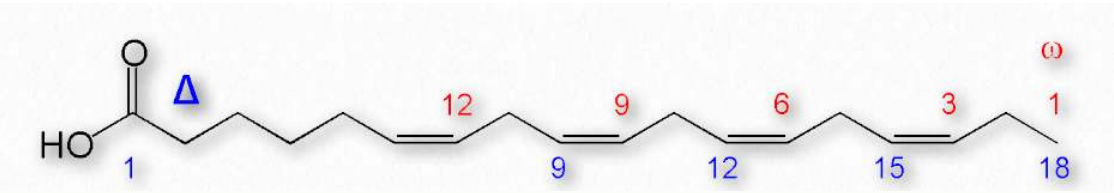


Figure 2.195 - Δ and ω numbering systems for fatty acids Image by Pehr Jacobson

Fatty acids are described as essential fatty acids if they must be in the diet (can't be synthesized by the organism) and nonessential fatty acids if the organism can synthesize them. Humans and other animals lack the desaturase enzymes necessary to make double bonds at positions greater than Δ-9, so fatty acids with double bonds beyond this position must be obtained in the diet. Linoleic acid and linolenic acid, both fall in this category. Related unsaturated fatty acids can be made from these fatty acids, so the presence of linoleic and linolenic acids in the diet eliminates the need to have all unsaturated fatty acids in the diet. Both linoleic and linolenic acid contain 18 carbons, but linoleic acid is an ω-6 fatty acid, whereas linolenic acid is an ω-3 fatty acid. Notably, ω-6 fatty acids tend to be proinflammatory, whereas ω-3 fatty acids are lesser so.

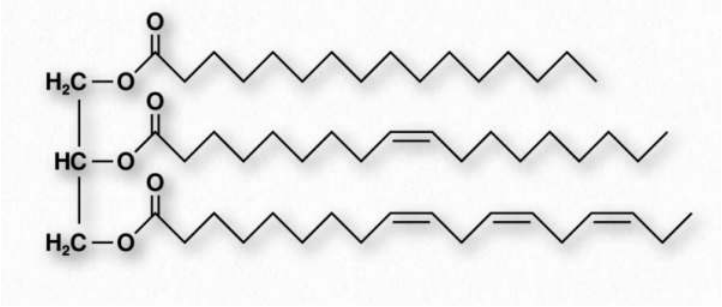


Figure 2.196 - Structure of a fat/oil

Fats/oils

Fats and oils are the primary energy storage forms of animals and are also known as triacylglycerols and triglycerides, since they consist of a glycerol molecule linked via ester bonds to three fatty acids (Figure 2.196). Fats and oils have the same basic structure. We give the name fat to those compounds that are solid at room temperature and the name oil to those that are liquid at room temperature. Note that biological oils are not the same as petroleum oils.

Increasing the number of unsaturated fatty acids (and the amount of unsaturation in a given fatty acid) in a fat decreases the melting temperature of it. Organisms like fish, which live in cool environments, have fats with more unsaturation and this is why fish oil contains polyunsaturated fatty acids.

Adipocytes

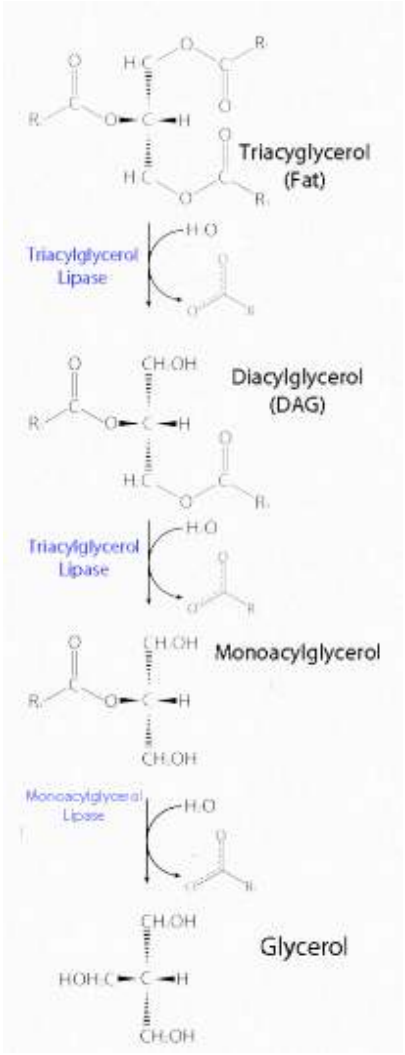


Figure 2.197 - Lipase action on a fat Image by Aleia Kim

Fats are stored in the body in specialized cells known as adipocytes. Enzymes known as lipases release fatty acids from fats by hydrolysis reactions (Figure 2.197). Triacylglycercol lipase (pancreatic - Figure 2.198) is able to cleave the first two fatty acids from the fat. A second enzyme, monoacylglycerol lipase, cleaves the last fatty acid. Fats can be synthesized by replacing the phosphate on phosphatidic acid with a fatty acid.

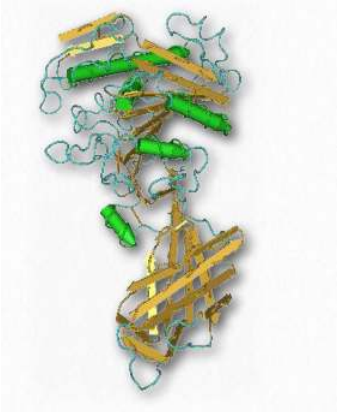


Figure 2.198 - Pancreatic lipase

Glycerophospholipids

Glycerophospholipids (phosphoglycerides) are important components of the lipid bilayer of cellular membranes. Phosphoglycerides are structurally related to fats, as both are derived from phosphatidic acid (Figure 2.199). Phosphatidic acid is a simple glycerophospholipid that is usually converted into phosphatidyl compounds. These are made by esterifying various groups, such as ethanolamine, serine, choline, inositol, and others (Figure 2.200) to the phosphate of phosphatidic acid. All of these compounds form lipid bilayers in aqueous solution , due to their amphiphilic nature.

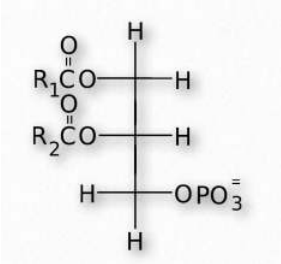


Figure 2.199 - Structure of phosphatidic acid. R1 and R2 are alkyl groups of fatty acids.

Phosphatidylethanolamines

Since all glycerolipids can have a variety of fatty acids at positions 1 and 2 on the glycerol, they all are families of compounds. The phosphatidylethanolamines are found in all living cells and are one of the most common phosphatides, making up about 25% of them. They are common constituents of brain tissue and in the spinal cord, making up as much as 45% of the total phospholipids. Phosphatidylethanolamines are asymmetrically distributed across membranes, being preferentially located on the inner leaflet (closest to the cytoplasm) of the plasma membrane. Metabolically, phosphatidylethanloamines are precursors of phosphatidylcholines. Phosphatidylserines Phosphatidylserines are another group of phosphatidyl compounds that are preferentially distributed across the lipid bilayer of the plasma membrane. Like the phosphatidylethanolamines, phosphatidylserines are preferentially located on the inner leaflet of the plasma membrane. When apoptosis (cell suicide) occurs, the preferential distribution is lost and the phosphatidylserines appear on the outer leaflet where they serve as a signal to macrophages to bind and destroy the cell.

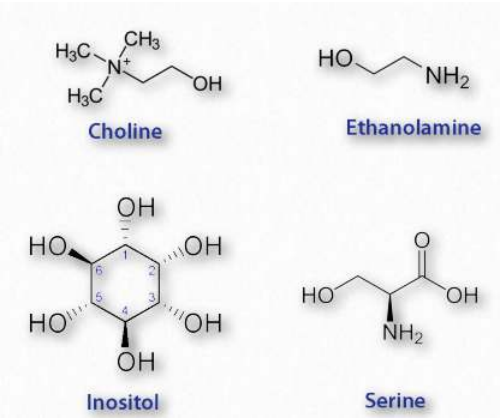


Figure 2.200 - Four common components of phosphatides Wikipedia

Phosphatidylcholines

Phosphatidylcholines (Figure 2.201) are another group of important membrane components. They tend to be found more commonly on the outer leaflet of the plasma membrane. Nutritionally, the compounds are readily obtained from eggs and soybeans. Phosphatidylcholines are moved across membranes by Phosphatidylcholine transfer protein (PCTP). This protein, which is sensitive to the levels of phosphatidylcholines, acts to stimulate the activity of a thioesterase (breaks thioester bonds, such as acyl-CoAs) and activates PAX3 transcription factors.

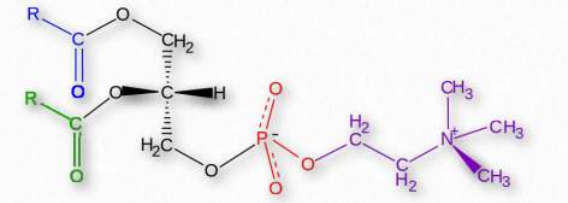


Figure 2.201 - Phosphatidylcholine

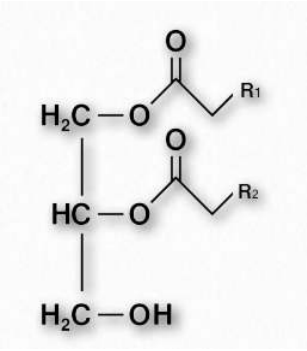


Figure 2.204 - Diacylglycerol

Diacylglycerol

Diacylglycerol (also called diglyceride and DAG - Figure 2.204) is an important intermediate in metabolic pathways. It is produced, for example, in the first step of the hydrolysis of fat and is also produced when membrane lipids, such as PIP2 (phosphatidylinositol-4,5-bisphosphate) are hydrolyzed by phospholipase C in a signaling cascade.

DAG is itself a signaling compound, binding to protein kinase C to activate it to phosphorylate substrates. Synthesis of DAG begins with glycerol-3-phosphate, which gains two fatty acids from two acyl-CoAs to form phosphatidic acid. Dephosphorylation of phosphatidic acid produces DAG. DAG can also be rephosphorylated by DAG kinase to re-make phosphatidic acid or another fatty acid can be added to make fat.

Signaling lipids

Inositol

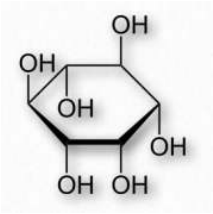


Figure 2.205 - Inositol

Though technically not a lipid itself, inositol is found in many lipids. Inositol is a derivative of cyclohexane containing six hydroxyl groups - one on each carbon (Figure 2.205. It has nine different stereoisomers of which one, cis-1,2,3,5-trans-4,6-cyclohexanehexol (called myo-inositol) is the most common. It has a sweet taste (half that of sucrose).

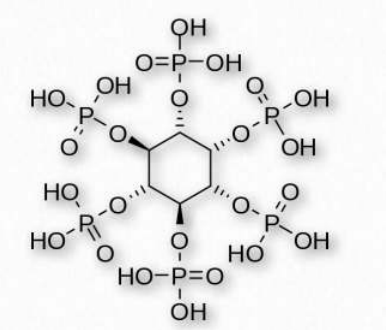


Figure 2.206 - Phytic acid

Numerous phosphorylated forms of the compound exist, from a single phosphate to six (one on each carbon). Phytic acid, for example, in plants, has six phosphates (Figure 2.206) that it uses to store phosphate. Inositol is produced from glucose and was once considered vitamin B8, but is made by the body in adequate amounts, so it is not now considered a vitamin. Phosphorylated forms of inositol are found in phosphoinositides, such as PIP2 and PIP3, both of which are important in signaling processes. Some of these include insulin signaling, fat catabolism, calcium regulation, and assembly of the cytoskeleton.

Phosphoinositides

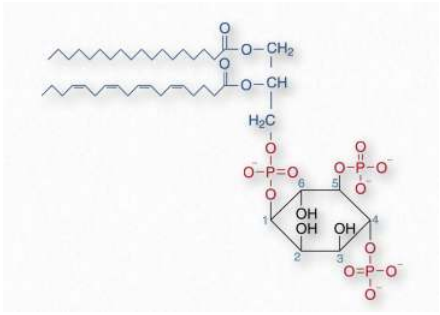


Figure 2.207 - Structure of PIP_2

Compounds based on phosphatidylinositol (PI) are often called phosphoinositides. These compounds have important roles in signaling and membrane trafficking. Hydroxyls on carbons 3,4, and 5 of the inositol ring are targets for phosphorylation by a variety of kinases. Seven different combinations are used. Steric hindrance inhibits phosphorylation of carbons 2 or 6. Naming of these phosphorylated compounds follows generally as PI(#P)P, PI(#P, #P)P, or PI(#P, #P, #P)P where #P refers to the number of the carbon where a phosphate is located. For example, PI(3)P refers to a phosphatidyl compound with a phosphate added to carbons 3 of the inositol ring, whereas PI(3,4,5)P is a phosphatidyl compound with a phosphate added to carbons 3,4,and 5.

Phosphatidylinositol-4,5- bisphosphate

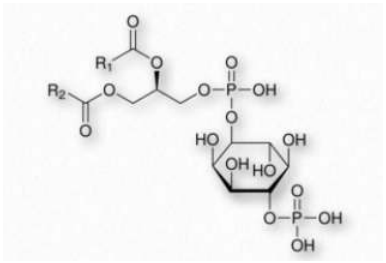


Figure 2.208 - Phosphatidylinositol-4- phosphate

Phosphatidylinositol-4,5-bisphosphate (PIP2 - Figure 2.207) is a phospholipid of plasma membranes that functions in the phospholipase C signaling cascade. In this signaling pathway, hydrolysis catalyzed by phospholipase C releases inositol-1,4,5-trisphosphate (IP3) and diacylglycerol. Synthesis of PIP2 begins with phosphatidylinositol, which is phosphorylated at position 4 followed by phosphorylation at position 5 by specific kinases.

PIP2 can be phosphorylated to form the signaling molecule known as phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Along with PIP3, PIP2 serves as a docking phospholipid for the recruitment of proteins that play roles in signaling cascades. Binding of PIP2 is

also required by inwardly directed potassium channels.

Phosphatidylinositol (3,4,5)- trisphosphate

Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) is an important molecule for the activation of signaling proteins, such as AKT, which activates anabolic signaling pathways related to growth and survival. PIP3 can be dephosphorylated by phosphatase PTEN to yield PIP2 and can be synthesized from PIP2 by kinase action of Class I PI 3- kinases. Kinase activity to synthesize PIP3 results in movement of PIP3-binding proteins to the plasma membrane. They include Akt/ PKB, PDK1, Btk1, and ARNO and each is activated by binding to PIP3.

Eicosanoids

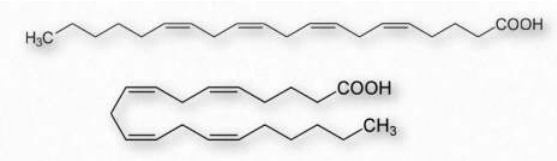


Figure 2.213 - Arachidonic acid drawn as straight (top) and bent (bottom)

Fatty acids made from omega-6 and omega-3 fatty acids include three important fatty acids containing 20 carbons. They include arachidonic acid (an ω-6 fatty acid with four double bonds (Δ-5,8,11,14) - Figure 2.213), eicosapentaenoic acid (an ω-3 fatty acid with five double bonds, and dihomo-γ-linolenic acid (an ω-6 fatty acid with three double bonds). The class of compounds known as eicosanoids is made by oxidation of these compounds. Subclasses include include prostaglandins, prostacyclins, thromboxanes, lipoxins, leukotrienes, and endocannabinoids (Figures 2.214-2.219). Eicosanoids play important roles affecting inflammation, immunity, mood, and behavior.

Prostaglandins

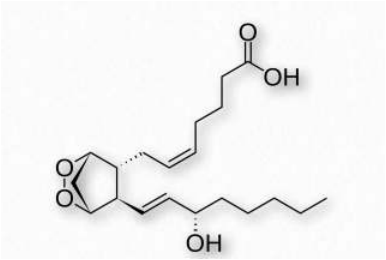


Figure 2.214 - Prostaglandin PGH_2

A collection of molecules acting like hormones, prostaglandins are derived from arachidonic acid and have many differing (even conflicting) physiological effects. These include constriction or dilation of vascular smooth muscle cells, induction of labor, regulation of inflammation, and action on the thermoregulatory center of the hypothalamus to induce fever, among others.

Prostaglandins are grouped with the thromboxanes (below) and prostacyclins (below), as prostanoids. The prostanoids, which all contain 20 carbons are a subclass of the eicosanoids. Prostaglandins are found in most tissues of higher organisms. They are autocrine or paracrine compounds produced from essential fatty acids. The primary precursor of the prostaglandins is the fatty acid known as arachidonic acid and the prostaglandin made from it is known as PGH2 (Figure 2.214), which, in turn is a precursor of other prostaglandins, as well as the prostacyclins and thromboxanes.

Interesting prostaglandins

PGD_2 - inhibits hair follicle growth, vasodilator, causes bronchial constriction, higher in lungs of asthmatics than others.

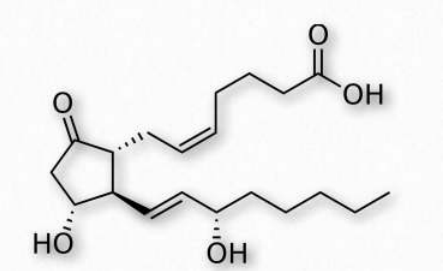


Figure 2.215 Prostaglandin E

PGE_2 (Figure 2.215) - exerts effects in labor (soften cervix, uterine contraction), stimulates bone resorption by osteoclasts, induces fever, suppresses T-cell receptor signaling, vasodilator, inhibits release of noradrenalin from sympathetic nerve terminals. It is a potent activator of the Wnt signaling pathway.

A prostaglandin can have opposite effects, depending on which receptor it binds to. Binding of PGE2 to the EP1 receptor causes bronchoconstriction and smooth muscle contraction, whereas binding of the same molecule to the EP2 receptor causes bronchodilation and smooth muscle relaxation.

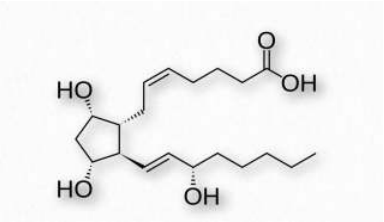


Figure 2.216 - Prostaglandin $F_{2\alpha}$

$PGF_{2\alpha}$ (Figure 2.216)- uterine contractions, induces labor, bronchoconstriction.

PGI_2 - vasodilation, bronchodilation, inhibition of platelet aggregation.

Thromboxanes

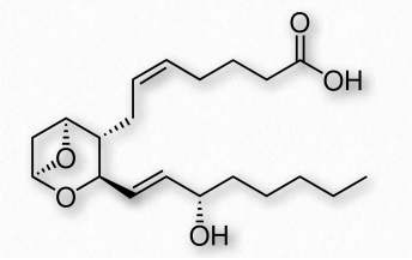


Figure 2.217 Thromboxane $A2_2$

Thromboxanes play roles in clot formation and named for their role in thrombosis. They are potent vasoconstrictors and facilitate platelet aggregation. They are synthesized in platelets, as well. The anti-clotting effects of aspirin have their roots in the inhibition of synthesis of PGH2, which is the precursor of the thromboxanes. The most common thromboxanes are A2 (Figure 2.217) and B2.

Leukotrienes

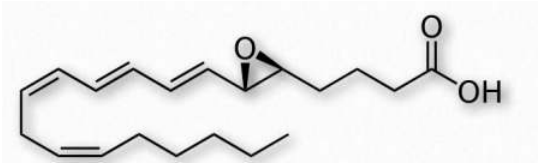


Figure 2.219 - Leukotriene A_4 (LTA_4)

Another group of eicosanoid compounds are the leukotrienes (Figure 2.219). Like prostaglandins, leukotrienes are made from arachidonic acid. The enzyme catalyzing their formation is a dioxygenase known as arachidonate 5-lipoxygenase. Leukotrienes are involved in regulating immune responses. They are found in leukocytes and other immunocompetent cells, such as neutrophils, monocytes, mast cells, eosinophils, and basophils. Leukotrienes are associated with production of histamines and prostaglandins, which act as mediators of inflammation. Leukotrienes also trigger contractions in the smooth muscles of the bronchioles. When overproduced, they may pay a role in asthma and allergic reactions. Some treatments for asthma aim at inhibiting production or action of leukotrienes.

Steroids: membrane components and hormones

Steroids, such as cholesterol are found in membranes and act as signaling hormones in traveling through the body.

Cholesterol

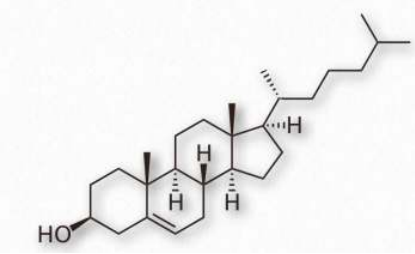


Figure 2.220 - Structure of cholesterol

Arguably, no single biomolecule has generated as much discussion and interest as has cholesterol (Figure 2.220). Certainly, from the perspective of the Nobel Prize committee, no small molecule even comes close, with 13 people having been awarded prizes for work on it. Evidence for cholesterol's importance comes from the study of brain tissue where it comprises 10-15% of the dry mass.

Membrane flexibility

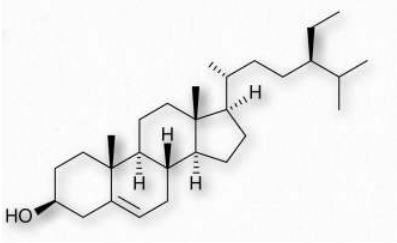


Figure 2.221 - Sitosterol - A phytosterol

In animal cells, cholesterol provides for membrane flexibility that allows for cellular movement that is in contrast to plant and bacterial cells with fixed structures. Cholesterol is made in many cells of the body, with the liver making the greatest amount. The anabolic pathway leading to synthesis of cholesterol is known as the isoprenoid pathway and branches of it lead to other molecules including other fat-soluble vitamins.



Figure 2.222 - Margarine - A common source of trans fat Wikipedia

Cholesterol is only rarely found in prokaryotes (Mycoplasma, which requires it for growth, is an exception) and is found in only trace amounts in plants. Instead, plants synthesize similar compounds called phytosterols (Figure 2.221). On average, the body of a

150 pound adult male makes about 1 gram of cholesterol per day, with a total content of about 35 grams.

Packaging

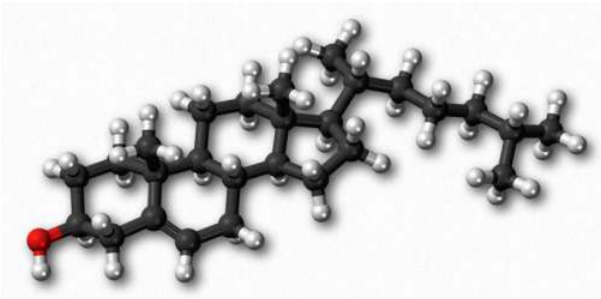


Figure 2.223 - Cholesterol - Ball and stick model

Cholesterol's (and other lipids') hydrophobicity requires special packaging into lipoprotein complexes (called chylomicrons, VLDLs, IDLs, LDLs, and HDLs) for movement in the lymph system and bloodstream. Though cholesterol can be made by cells, they also take it up from the blood supply by absorbing cholesterol-containing LDLs directly in a process called receptor-mediated endocytosis.

Oxidative damage to LDLs can lead to formation of atherosclerotic plaques and this is why cholesterol has gotten such a negative image in the public eye. The liver excretes cholesterol through the bile for elimination into the digestive system, but the compound is recycled there.

Reducing cholesterol levels

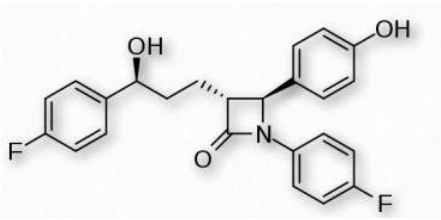


Figure 2.224 - Ezetimibe - An inhibitor of cholesterol absorption

Strategies for reducing cholesterol in the body focus primarily on three areas - reducing consumption, reducing endogenous synthesis, and reducing the recycling. Dietary considerations, such as saturated fat versus unsaturated fat consumption are currently debated. Dietary trans fats, though, correlate with incidence of coronary heart disease. Consumption of vegetables may provide some assistance with reducing levels of cholesterol recycled in the digestive system, because plant phytosterols compete with cholesterol for reabsorption and when this happens, a greater percentage of cholesterol exits the body in the feces. Drugs related to penicillin are also used to inhibit cholesterol recycling. One of these is ezetimibe, shown in Figure 2.224.

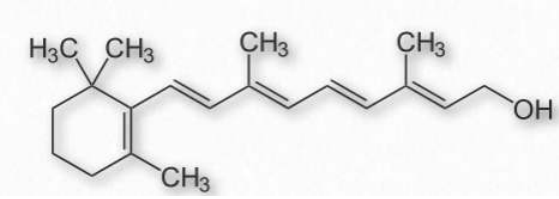


Figure 2.225 - All-trans retinol

Genetic defects in the cholesterol movement system are a cause of the rare disease known as familial hypercholesterolemia in which the blood of afflicted individuals contains dangerously high levels of LDLs. Left untreated, the disease is often fatal in the first 10-20 years of life. While LDLs have received (and deserve) a bad rap, another group of lipoprotein complexes known as the HDLs (high density lipoprotein complexes) are known as "good cholesterol" because their levels correlate with removal of debris (including cholesterol) from arteries and reduce inflammation.

Membrane function

In membranes, cholesterol is important as an insulator for the transmission of signals in nerve tissue and it helps to manage fluidity of membranes over a wide range of temperatures. Stacked in the lipid bilayer, cholesterol decreases a membrane's fluidity and its permeability to neutral compounds, as well as protons and sodium ions. Cholesterol may play a role in signaling by helping with construction of lipid rafts within the cell membrane.

Steroid Hormones

Steroid hormones are all made from cholesterol and are grouped into five categories - mineralocorticoids (21 carbons), glucocorticoids (21 carbons), progestagens (21 carbons), androgens (19 carbons), and estrogens (18 carbons).

Mineralocorticoids

Mineralocorticoids are steroid hormones that influence water and electrolyte balances. Aldosterone (Figure 2.238) is the primary mineralocorticoid hormone, though other steroid hormones (including progesterone) have some functions like it. Aldosterone stimulates kidneys to reabsorb sodium, secrete potassium, and passively reabsorb water. These actions have the effect of increasing blood pressure and blood volume. Mineralocorticoids are produced by the zona glomerulosa of the cortex of the adrenal gland.

Glucocorticoids

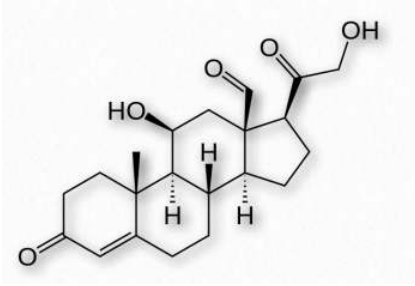


Figure 2.238 - Aldosterone - A mineralocorticoid

Glucocorticoids (GCs) bind to glucocorticoid receptors found in almost every vertebrate animal cell and act in a feedback mechanism in the immune system to reduce its activity. GCs are used to treat diseases associated with overactive immune systems. These include allergies, asthma, and autoimmune dis- Figure 2.237 - Steroid numbering scheme Image by Pehr Jacobson eases. Cortisol (Figure 2.239) is an important glucocorticoid with cardiovascular, metabolic, and immunologic functions. The synthetic glucocorticoid known as dexamethasone has medical applications for treating rheumatoid arthritis, bronchospasms (in asthma), and inflammation due to its increased potency (25-fold) compared to cortisol. Glucocorticoids are produced primarily in the zona fasciculata of the adrenal cortex.

Progestagens

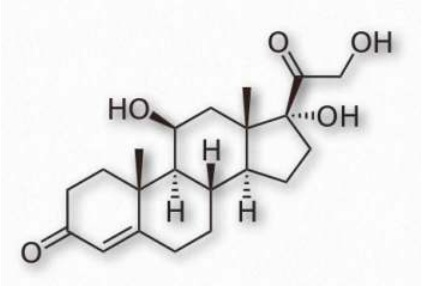


Figure 2.239 - Cortisol - A glucocorticoid

Progestagens (also called gestagens) are steroid hormones that work to activate the progesterone receptor upon binding to it. Synthetic progestagens are referred to as progestins. The most common progestagen is progesterone (also called P4 - Figure 2.240) and it has functions in maintaining pregnancy. Progesterone is produced primarily in the diestrus phase of the estrous cycle by the corpus luteum of mammalian ovaries. In pregnancy, the placenta takes over most progesterone production.

Androgens

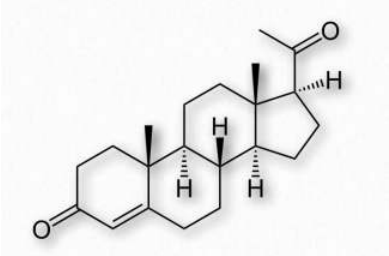


Figure 2.240 Progesterone - A progestagen

Androgens are steroid hormones that act by binding androgen receptors to stimulate development and maintenance of male characteristics in vertebrates. Androgens are precursors of estrogens (see below). The primary androgen is testosterone (Figure 2.241). Other important androgens include dihydrotestosterone (stimulates differentiation of penis, scrotum, and prostate in embryo) and androstenedione (common precursor of male and female hormones).

Estrogens

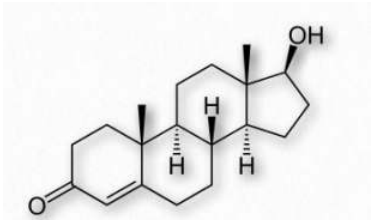


Figure 2.241 - Testosterone - An androgen

The estrogen steroid hormones are a class of compounds with important roles in menstrual and estrous cycles. They are the most important female sex hormones. Estrogens act by activating estrogen receptors inside of cells. These receptors, in turn, affect expression of many genes. The major estrogens in women include estrone (E1), estradiol (E2 - Figure 2.242), and estriol (E3). In the reproductive years, estradiol predominates. During pregnancy, estriol predominates and during menopause, estrone is the major estrogen.

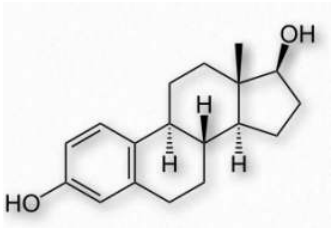


Figure 2.242 - Estradiol - An estrogen

Estrogens are made from the androgen hormones testosterone and androstenedione in a reaction catalyzed by the enzyme known as aromatase. Inhibition of this enzyme with aromatase inhibitors, such as exemestane, is a strategy for stopping estrogen production. This may be part of a chemotherapeutic treatment when estrogenresponsive tumors are present.

Lipid Transport: lipoprotein complexes and lipid movement in the body

Lipoprotein complexes are combinations of apolipoproteins and lipids bound to them that solubilize fats and other non-polar molecules, such as cholesterol, so they can travel in the bloodstream between various tissues of the body. The apolipoproteins provide the emulsification necessary for this. Lipoprotein complexes are formed in tiny “balls” with the water soluble apolipoproteins on the outside and non-polar lipids, such as fats, cholesteryl esters, and fat soluble vitamins on the inside.

They are categorized by their densities. These include (from highest density to the lowest) high density lipoproteins (HDLs), Low Density Lipoproteins (LDLs), Intermediate Density Lipoproteins (IDLs), Very Low Density Lipoproteins (VLDLs) and the chylomicrons. These particles are synthesized in the liver and small intestines.

Apolipoproteins

Name	Lipoprotein Complex(es)	Function
ApoA-I	HDL	Promotes Fat Movement to Liver
ApoA-II	HDL	Inhibit LCAT
ApoA-IV	Chylomicrons / HDL	Activate LCAT
AboB-48	Chylomicrons	Cholesterol Transport
AboB-100	VLDL / LDL	Bind LDL Receptor
ApoC-I	VLDL / LDL	Unknown
ApoC-II	All	Activate Lipoprotein Lipase
ApoC-III	All	Inhibit Lipoprotein Lipase
ApoD	HDL	Unknown
ApoE	VLDL / Chylomicrons / HDL	Clearance of Chylomicrons Remnants and VLDLs

Figure 2.256 - Apolipoproteins

Each lipoprotein complex contain a characteristic set of apolipoproteins, as shown in Figure 2.256. ApoC-II and ApoC-III are notable for their presence in all the lipoprotein complexes and the roles they play in activating (ApoC-II) or inactivating (ApoC-III) lipoprotein lipase. Lipoprotein lipase is a cellular enzyme that catalyzes the breakdown of fat from the complexes. ApoE (see below) is useful for helping the predict the likelihood of the occurrence of Alzheimer's disease in a patient.

Deep dive: example of mRNA editing

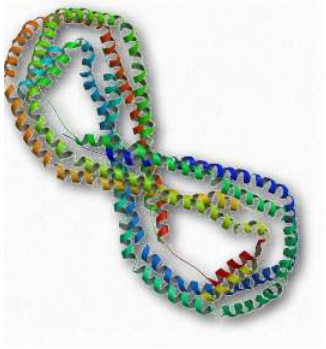


Figure 2.257 - ApoA-I

ApoB-48 and ApoB-100 are interesting in being coded by the same gene, but a unique mRNA sequence editing event occurs that converts one into the other. ApoB-100 is made in the liver, but ApoB-48 is made in the small intestine. The small intestine contains an enzyme that deaminates the cytidine at nucleotide #2153 of the common mRNA. This changes it to a uridine and changes the codon it is in from CAA (codes for glutamine) to UAA (stop codon). The liver does not contain this enzyme and does not make the change in the mRNA. Consequently, a shorter protein is synthesized in the intestine (ApoB-48) than the one that is made in the liver (ApoB-100) using the same gene sequence in the DNA.

Movement

The movement of fats in the body is important because they are not stored in all cells. Only specialized cells called adipocytes store fat. There are three relevant pathways in the body for moving lipids. As described below, they are 1) the exogenous pathway (for fats that come in as food); 2) the endogenous pathway (for lipids that are synthesized in the body), and 3) the reverse transport pathway.

Exogenous pathway

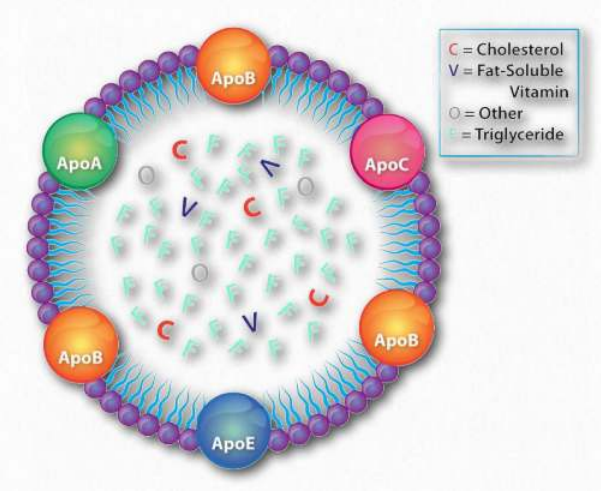


Figure 2.258 - Schematic diagram of a chylomicron Image by Aleia Kim

Dietary fat entering the body from the intestinal system must be transported, as appropriate, to places needing it or storing it. This is the function of the exogenous pathway of lipid movement in the body. All dietary lipids (fats, cholesterol, fat-soluble vitamins, and other lipids) are moved by it. In the case of dietary fat, it begins its journey after ingestion first by being solubilized by bile acids in the intestinal tract. After passing through the stomach, pancreatic lipases clip two fatty acids from the fat, leaving a monoacyl glycerol. The fatty acids and monoacyl glycerol are absorbed by intestinal cells (enterocytes) and reassembled back into a fat, and then this is mixed with phospholipids, cholesterol esters, and apolipoprotein B-48 and processed to form chylomicrons (Figures 2.258 & 2.259) in the Golgi apparatus and smooth endoplasmic reticulum.

Exocytosis

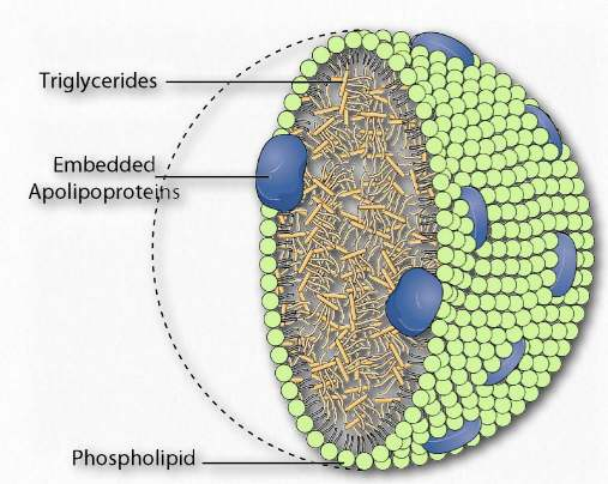


Figure 2.259 - Another perspective of a chylomicron WIkipedia

These are exocytosed from the cell into lymph capillaries called lacteals. The chylomicrons pass through the lacteals and enter the bloodstream via the left subclavian vein. Within the bloodstream, lipoprotein lipase breaks down the fats causing the chylomicron to shrink and become what is known as a chylomicron remnant. It retains its cholesterol and other lipid molecules.

The chylomicron remnants travel to the liver where they are absorbed (Figure 2.260). This is accomplished by receptors in the liver that recognize and bind to the ApoE of the chylomicrons. The bound complexes are then internalized by endocytosis, degraded in the lysosomes, and the cholesterol is disbursed in liver cells.

Endogenous pathway

The liver plays a central role in managing the body's needs for lipids. When lipids are needed by the body or when the capacity of the liver to contain more lipids than is supplied by the diet, the liver packages up fats and cholesteryl esters into Very Low Density Lipoprotein (VLDL) complexes and exports them via the endogenous pathway. VLDL complexes contain ApoB-100, ApoC-I,

ApoC-II, ApoC-III, and ApoE apolipoproteins. VLDLs enter the blood and travel to muscles and adipose tissue where lipoprotein lipase is activated by ApoC-II. In the muscle cells, the released fatty acids are taken up and oxidized. By contrast, in the adipoctyes, the fatty acids are taken up and reassembled back into triacylglycerides (fats) and stored in fat droplets. Removal of fat from the VLDLs causes them to shrink, first to Intermediate Density Lipoprotein (IDL) complexes (also called VLDL remnants) and then to Low Density Lipoprotein (LDL) complexes.

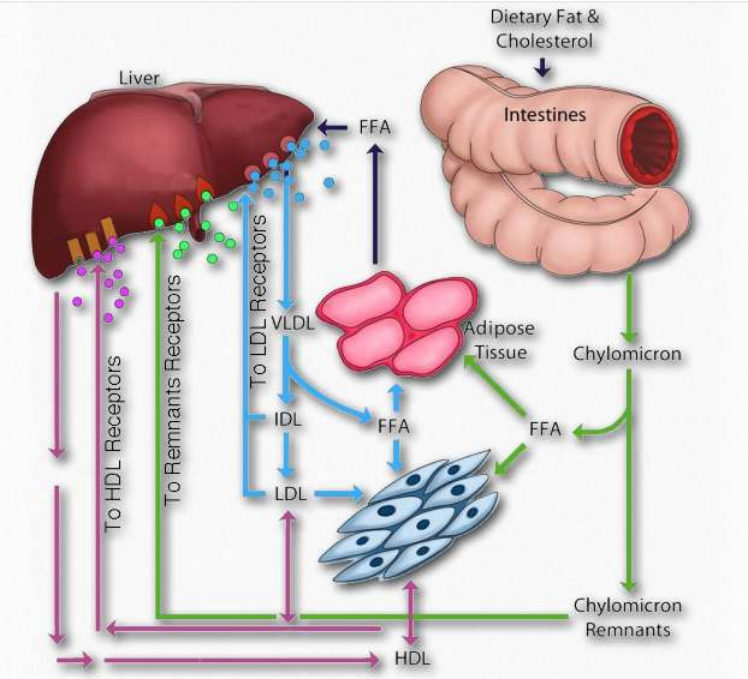


Figure 2.260 - Movement of lipids in the body - Green = exogenous pathway; Blue = endogenous pathway; Purple = reverse transport pathway Image by Aleia Kim

Shrinking of VLDLs is accompanied by loss of apolipoproteins so that LDLs are comprised primarily of ApoB-100. This lipoprotein complex is important because cells have receptors for it to bind and internalize it by receptor-mediated endocytosis (Figure 2.261). Up until this point, cholesterol and cholesteryl esters have traveled in chylomicrons, VLDLs, and IDLs as fat has been stripped stripped away. For cholesterol compounds to get into the cell from the lipoprotein complexes, they must be internalized by cells and that is the job of receptormediated endocytosis.

Reverse transport pathway

Another important consideration of the movement of lipids in the body is the reverse transport pathway (Figure 2.260). It is also called the reverse cholesterol transport pathway, since cholesterol is the primary molecule involved. This pathway involves the last class of lipoprotein complexes known as the High Density Lipoproteins (HDLs). In contrast to the LDLs, which are commonly referred to as “bad cholesterol” (see below also), the HDLs are known as “good cholesterol.”

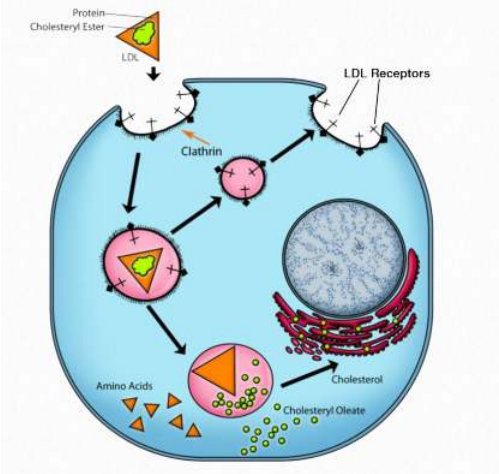


Figure 2.261 - The process of receptor-mediated endocytosis Image by Aleia Kim

HDLs are synthesized in the liver and small intestine. They contain little or no lipid when made (called depleted HDLs), but serve the role of “scavenger” for cholesterol in the blood and from remnants of other (damaged) lipoprotein complexes in the blood. To perform its task, HDLs carry the enzyme known as lecithincholesterol acyl transferase (LCAT), which they use to form cholesteryl esters using fatty acids from lecithin (phosphatidylcholine) and then they internalize them.

The cholesterol used for this purpose comes from the bloodstream, from macrophages, and from foam cells (macrophage-LDL complexes - Figure 2.262). Addition of cholesteryl esters causes the HDL to swell and Figure 2.261 - The process of receptor-mediated endocytosis Image by Aleia Kim when it is mature, it returns its load of cholesterol back to the liver or, alternatively, to LDL molecules for endocytosis. HDLs have the effect of lowering levels of cholesterol and it is for that reason they are described as “good cholesterol.”

Regulation of lipid transfer

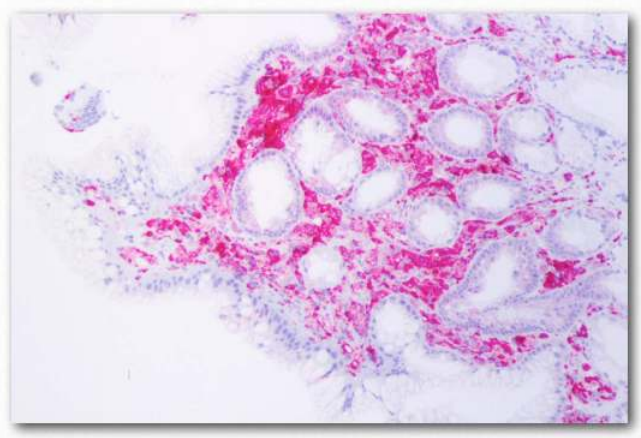


Figure 2.262 - Foam cell aggregate Wikipedia

It is important that cells get food when they need it so some control of the movement of nutrients is critical. The liver, which plays the central role in modulating blood glucose levels, is also important for performing the same role for lipids. It accomplishes this task the use of specialized LDL receptors on its surface. Liver LDL receptors bind LDLs that were not taken up by other cells in their path through the bloodstream. High levels of LDLs are a signal to the liver to reduce the creation of VLDLs for release.

People with the genetic disease known as familial hypercholesterolemia, which manifests with dangerously high levels of LDLs, lack properly functioning LDL receptors on their liver cells.Figure 2.263 - Progression of atherosclerosis Wikipedia Figure 2.263 - Progression of atherosclerosis Wikipedia Figure 2.263 - Progression of atherosclerosis Wikipedia

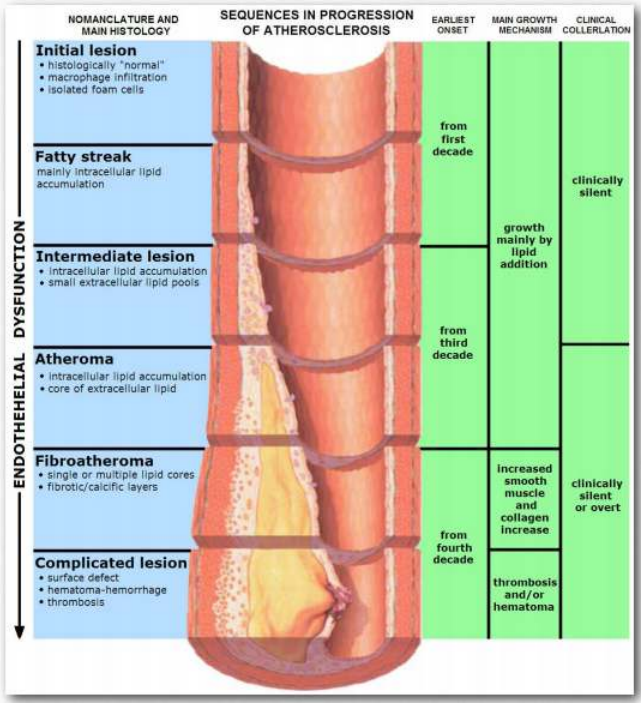


Figure 2.263 - Progression of atherosclerosis Wikipedia

In sufferers of this disease, the liver never gets the signal that the LDL levels are high. In fact, to the liver, it appears that all VLDLs and LDLs are being taken up by peripheral tissues, so it creates more VLDLs to attempt to boost levels. Untreated, the disease used to be fatal early, but newer drugs like the statins have significantly increased life spans of patients. Cellular needs for the contents of LDLs are directly linked to the levels of synthesis of LDL receptors on their membranes. As cells are needing more cholesterol, their synthesis of components for receptors goes up and it decreases as need diminishes.

Good cholesterol / bad cholesterol

It is commonly accepted that “high cholesterol” levels are not healthy. This is due, at least indirectly, to the primary carriers of cholesterol, the LDLs. A primary function of the LDLs is to deliver cholesterol and other lipids directly into cells by receptor mediated endocytosis (Figure 2.237). High levels of LDLs, though, are correlated with formation of atherosclerotic plaques (Figure 2.263 & 2.264) and incidence of atherosclerosis, leading to the description of them as “bad cholesterol.” This is because when LDL levels are very high, plaque formation begins. It is thought that reactive oxygen species (higher in the blood of smokers) causes partial oxidation of fatty acid groups in the LDLs. When levels are high, they tend to accumulate in the extracellular matrix of the epithelial cells on the inside of the arteries. Macrophages of the immune system take up the damaged LDLs (including the cholesterol).

Since macrophages can’t control the amount of cholesterol they take up, cholesterol begins to accumulate in them and they take on appearance that leads to their being described as “foam cells.” With too much cholesterol, the foam cells, however, are doomed to die by the process of programmed cell death (apoptosis). Accumulation of these, along with scar tissue from inflammation result in formation of a plaque. Plaques can grow and block the flow of blood or pieces of them can break loose and plug smaller openings in the blood supply, ultimately leading to heart attack or stroke.

Good cholesterol

On the other hand, high levels of HDL are inversely correlated with atherosclerosis and arterial disease. Depleted HDLs are able to remove cholesterol from foam cells. This occurs as a result of contact between the ApoA-I protein of the HDL and a transport protein on the foam cell (ABC-G1). Another transport protein in the foam cell, ABCA-1 transports extra cholesterol from inside the cell to the plasma membrane where it is taken up into the HDL and returned to the liver or to LDLs by the reverse transport cholesterol pathway.



Figure 2.264 - Actual carotid artery plaque Wikipedia

Deficiency of the ABCA-1 gene leads to Tangier disease. In this condition, HDLs are almost totally absent because they remain empty as a result of not being able to take up cholesterol from foam cells, so they are destroyed by the body.

ApoE and Alzheimer's disease

ApoE is a component of the chylomicrons and is also found in brain, macrophages, kidneys, and the spleen. In humans, it is found in three different alleles, E2, E3, and E4. The E4 allele (present at about 14% of the population) is associated with increased likelihood of contracting Alzheimer's disease. People heterozygous for the allele are 3 times as likely to contract the disease and those homozygous for it are 15 times as likely to do so. It is not known why this gene or allele is linked to the disease. The three alleles differ only slightly in amino acid sequence, but the changes do cause notable structural differences. The E4 allele is associated with increased calcium ion levels and apoptosis after injury. Alzheimer's disease is associated with accumulation of aggregates of the β- amyloid peptide. ApoE does enhance the proteolytic breakdown of it and the E4 isoform is not as efficient in these reactions as the other isoforms.

This page titled 5.2: Structure and Function - Lipids and Their Transport is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **2.8: Structure and Function - Lipids and Membranes** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

5.2.1: 5.2.1 Optional- other interesting lipids

Source: BiochemFFA_2_7.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

Cardiolipins

Cardiolipins are an unusual set of glycerophospholipids in containing two diacylglycerol backbones joined in the middle by a diphosphoglycerol (Figure 2.202). It is an important membrane lipid, constituting about 20% of the inner mitochondrial membrane and is found in organisms from bacteria to humans. In both plants and animals, it is found almost totally in the inner mitochondrial membrane.

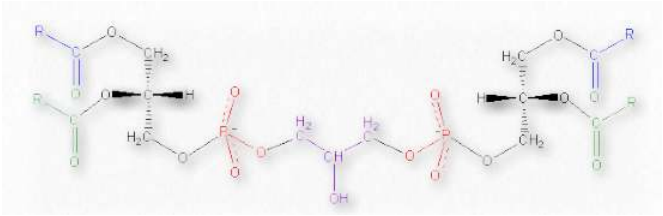


Figure 2.202 - Cardiolipin

The molecules appear to be required for both Complex IV and Complex III of the electron transport chain to maintain its structure. The ATP synthase enzyme (Complex V) of the oxidative phosphorylation system also binds four molecules of cardiolipin. It has been proposed that cardiolipin functions as a proton trap in the process of proton pumping by Complex IV.

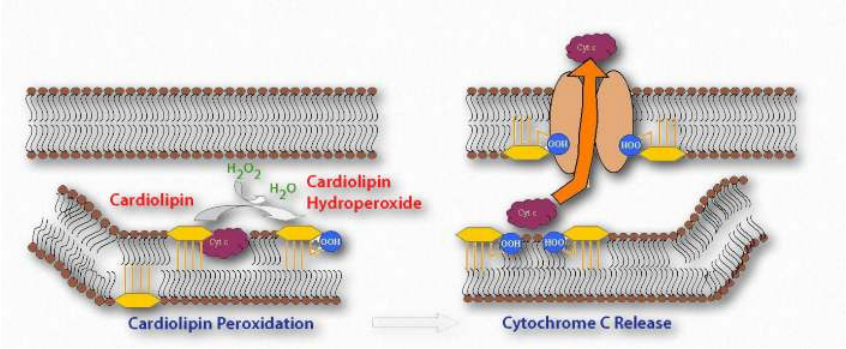


Figure 2.203 - Cardiolipin oxidation and the release of cytochrome C in apoptosis

Cardiolipin also plays a role in apoptosis. As shown in Figure 2.203, oxidation of cardiolipin by a cardiolipin-specific oxygenase causes cardiolipin to move from the inner mitochondrial membrane to the outer one, helping to form a permeable pore and facilitating the transport of cytochrome c out of the intermembrane space and into the cytoplasm - a step in the process of apoptosis.**Plasmalogens**

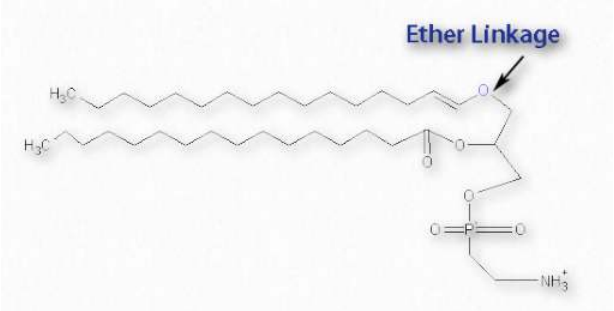


Figure 2.209 - Plasmalogen - A vinyl ether lipid Wikipedia

A special class of the glycerophospholipids are the plasmalogens (Figure 2.209). They differ in containing a vinyl ether linkage at position 1 of glycerol, in contrast to other glycerophopsholipids, which have an ester linkage at this position. Position 2 of each is

an ester. The precursor for the ether linkage is typically a 16 or 18 carbon saturated alcohol or an 18 carbon unsaturated alcohol.

At the phosphate tail, the most commonly attached groups are ethanolamine or choline. Plasmalogens are found abundantly in humans in heart (30-40% of choline phospholipids). 30% of the glycerophospholipids in brain are plasmalogens and 70% of the ethanolamine lipids of the myelin sheath of nerve cells are plasmalogens.

Though their function is not understood, it is believed that plasmalogens may provide some protection against reactive oxygen species and have roles in signaling.

Lecithin

Lecithin is a generic term for a combination of lipid substances that includes phosphoric acid, glycerol, glycolipids, triglycerides, and phospholipids. Lecithin is a wetting agent helpful with emulsification and encapsulation and is even used as an anti-sludge additive in motor lubricants. Lecithin is used in candy bars to keep cocoa and cocoa butter from separating. Though considered safe as a food ingredient, lecithin can be converted by gut bacteria to trimethylamine-N-oxide which may contribute to cholesterol deposition and atherosclerosis.

Sphingolipids

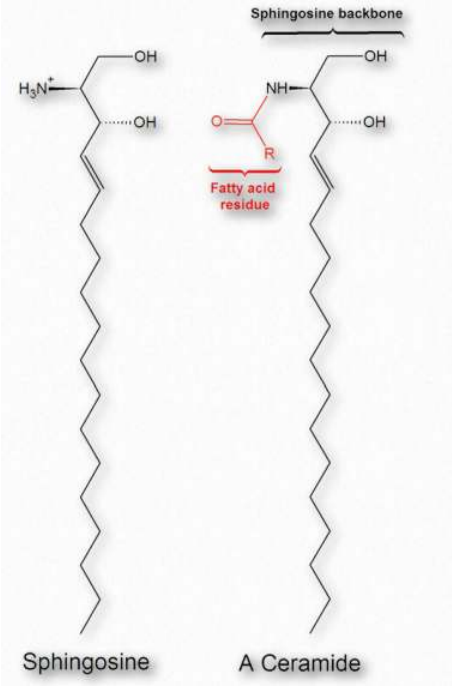


Figure 2.210 - Sphingosine and a ceramide made from it Wikipedia

Fatty acids are also components of a broad class of molecules called sphingolipids. Sphingolipids are structurally similar to glycerophospholipids, though they are synthesized completely independently of them starting with palmitic acid and the amino acid serine. Sphingolipids are named for the amino alcohol known as sphingosine (Figure 2.210), though they are not directly synthesized from it. Figure 2.211 shows the generalized structure of sphingolipids.

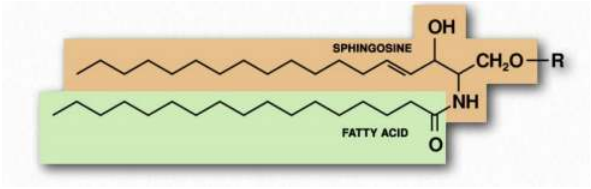


Figure 2.211 Schematic structure of a sphingolipid

If the R-group is a hydrogen, the molecule is called a ceramide. When the R-group is phosphoethanolamine the resulting molecule is sphingomyelin, an important component of the myelin sheath and lipid membranes. If a single, simple sugar is instead added, a cerebroside is created (Figure 2.212). Addition of a complex oligosaccharide creates a ganglioside.

Complex sphingolipids may play roles in cellular recognition and signaling. Sphingolipids are found most abundantly in plasma membrane and are almost completely absent from mitochondrial and endoplasmic reticulum membranes. In animals, dietary sphingolipids have been linked to reduced colon cancer, reductions in LDLs, and increases in HDLs. Like the glycerophospholipids, sphingolipids are amphiphilic. Most sphingolipids except sphingomyelin do not contain phosphate.

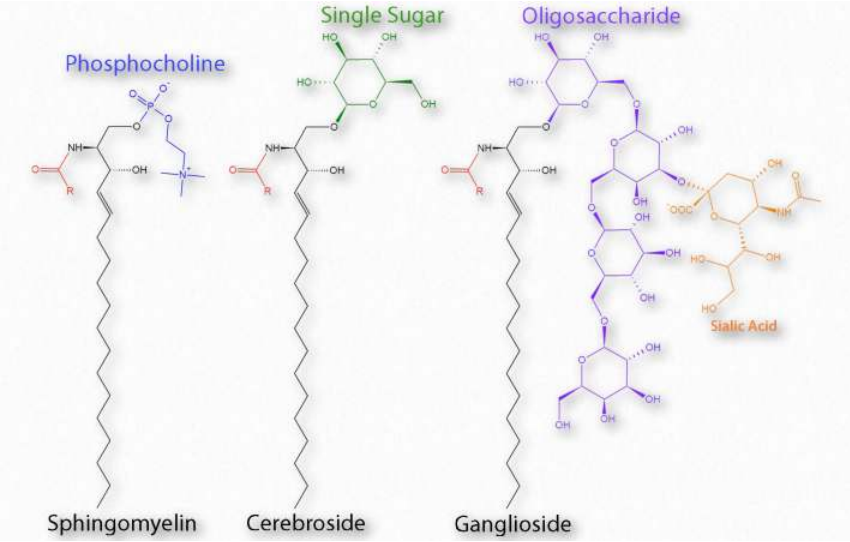


Figure 2.212 - Categories of Sphingolipid Wikipedia

Signaling lipids involved in inflammation

Eicosanoids

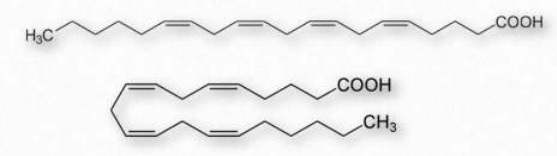


Figure 2.213 - Arachidonic acid drawn as straight (top) and bent (bottom)

Fatty acids made from omega-6 and omega-3 fatty acids include three important fatty acids containing 20 carbons. They include arachidonic acid (an ω-6 fatty acid with four double bonds (Δ-5,8,11,14) - Figure 2.213), eicosapentaenoic acid (an ω-3 fatty acid with five double bonds, and dihomo-γ-linolenic acid (an ω-6 fatty acid with three double bonds). The class of compounds known as eicosanoids is made by oxidation of these compounds. Subclasses include include prostaglandins, prostacyclins, thromboxanes, lipoxins, leukotrienes, and endocannabinoids (Figures 2.214-2.219). Eicosanoids play important roles affecting inflammation, immunity, mood, and behavior.

Prostaglandins

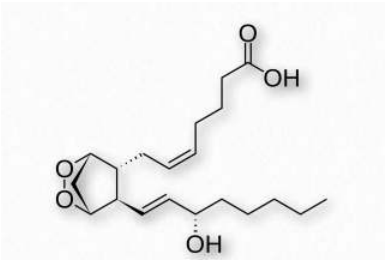


Figure 2.214 - Prostaglandin PGH_2

A collection of molecules acting like hormones, prostaglandins are derived from arachidonic acid and have many differing (even conflicting) physiological effects. These include constriction or dilation of vascular smooth muscle cells, induction of labor, regulation of inflammation, and action on the thermoregulatory center of the hypothalamus to induce fever, among others.

Prostaglandins are grouped with the thromboxanes (below) and prostacyclins (below), as prostanoids. The prostanoids, which all contain 20 carbons are a subclass of the eicosanoids. Prostaglandins are found in most tissues of higher organisms. They are autocrine or paracrine compounds produced from essential fatty acids. The primary precursor of the prostaglandins is the fatty acid known as arachidonic acid and the prostaglandin made from it is known as PGH2 (Figure 2.214), which, in turn is a precursor of other prostaglandins, as well as the prostacyclins and thromboxanes.

Interesting prostaglandins

PGD_2 - inhibits hair follicle growth, vasodilator, causes bronchial constriction, higher in lungs of asthmatics than others.

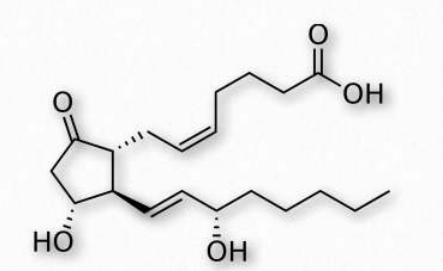


Figure 2.215 Prostaglandin E

PGE_2 (Figure 2.215) - exerts effects in labor (soften cervix, uterine contraction), stimulates bone resorption by osteoclasts, induces fever, suppresses T-cell receptor signaling, vasodilator, inhibits release of noradrenalin from sympathetic nerve terminals. It is a potent activator of the Wnt signaling pathway.

A prostaglandin can have opposite effects, depending on which receptor it binds to. Binding of PGE2 to the EP1 receptor causes bronchoconstriction and smooth muscle contraction, whereas binding of the same molecule to the EP2 receptor causes bronchodilation and smooth muscle relaxation.

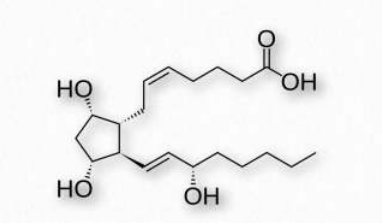


Figure 2.216 - Prostaglandin $F_{2\alpha}$

$PGF_{2\alpha}$ (Figure 2.216)- uterine contractions, induces labor, bronchoconstriction.

PGI_2 - vasodilation, bronchodilation, inhibition of platelet aggregation.

Thromboxanes

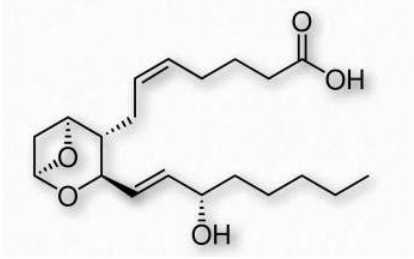


Figure 2.217 Thromboxane $A2_2$

Thromboxanes play roles in clot formation and named for their role in thrombosis. They are potent vasoconstrictors and facilitate platelet aggregation. They are synthesized in platelets, as well. The anti-clotting effects of aspirin have their roots in the inhibition of synthesis of PGH2, which is the precursor of the thromboxanes. The most common thromboxanes are A2 (Figure 2.217) and B2.

Prostacyclin

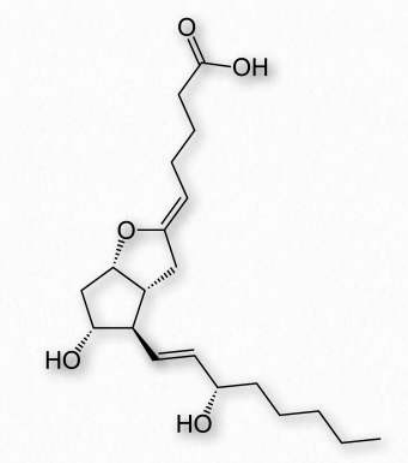


Figure 2.218 - Prostacyclin

Prostacyclin (also known as prostaglandin I2 or PGI2 - Figure 2.218) counters the effects of thromboxanes, inhibiting platelet activation and acting as vasodilators. It is produced from PGH2 by action of the enzyme prostacyclin synthase.

Leukotrienes

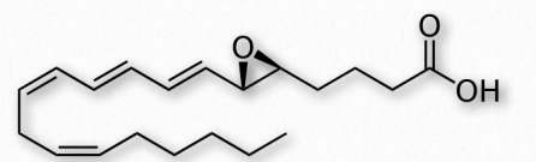


Figure 2.219 - Leukotriene A_4 (LTA_4)

Another group of eicosanoid compounds are the leukotrienes (Figure 2.219). Like prostaglandins, leukotrienes are made from arachidonic acid. The enzyme catalyzing their formation is a dioxygenase known as arachidonate 5-lipoxygenase. Leukotrienes are involved in regulating immune responses. They are found in leukocytes and other immunocompetent cells, such as neutrophils, monocytes, mast cells, eosinophils, and basophils. Leukotrienes are associated with production of histamines and prostaglandins, which act as mediators of inflammation. Leukotrienes also trigger contractions in the smooth muscles of the bronchioles. When overproduced, they may pay a role in asthma and allergic reactions. Some treatments for asthma aim at inhibiting production or action of leukotrienes.

Lipid-soluble vitamins are critical for enzyme function

Vitamin A

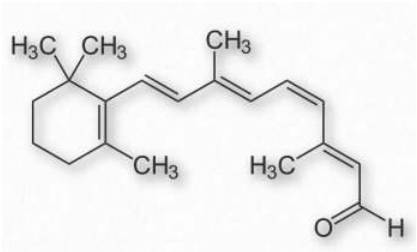


Figure 2.226 - 11-cis retinal

Vitamin A comes in three primary chemical forms, retinol (storage in liver - Figure 2.225), retinal (role in vision - Figure 2.226), and retinoic acid (roles in growth and development). All vitamin A forms are diterpenoids and differ only in the chemical form of

the terminal group. Retinol is mostly used as the storage form of the vitamin.

Retinol is commonly esterified to a fatty acid and kept in the liver. In high levels, the compound is toxic. Retinol is obtained in the body by hydrolysis of the ester or by reduction of retinal. Importantly, neither retinal nor retinol can be made from retinoic acid. Retinoic acid is important for healthy skin and teeth, as well as bone growth. It acts in differentiation of stem cells through a specific cellular retinoic acid receptor.

Sources

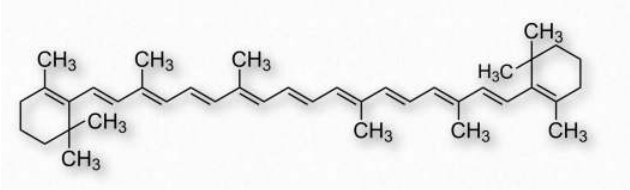


Figure 2.227 - β-Carotene

Good sources of vitamin A are liver and eggs, as well as many plants, including carrots, which have a precursor, β-carotene (Figure 2.227) from which retinol may be made by action of a dioxygenase.

Light sensitivity The conjugated double bond system in the side chain of vitamin A is sensitive to light and can flip between cis and trans forms on exposure to it. It is this response to light that makes it possible for retinal to have a role in vision in the rods and cones of the eyes. Here, the aldehyde form (retinal) is bound to the protein rhodopsin in the membranes of rod and cone cells.

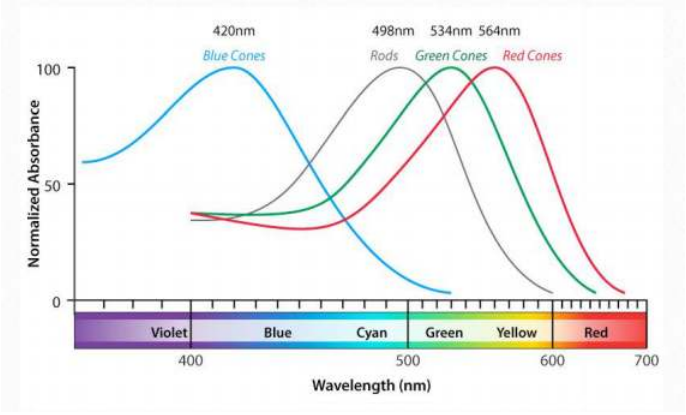


Figure 2.228 - Color sensitivity for cones and rods Image by Aleia Kim

When exposed to light of a particular wavelength, the “tail” of the retinal molecule will flip back and forth from cis to trans at the double bond at position 11 of the molecule. When this happens, a nerve signal is generated that signals the brain of exposure to light. Slightly different forms of rhodopsin have different maximum absorption maxima allowing the brain to perceive red, green and blue specifically and to assemble those into the images we see (Figure 2.228). Cones are the cells responsible for color vision, whereas rods are mostly involved in light detection in low light circumstances.

Deficiency and surplus

Deficiency of vitamin A is common in developing countries and was inspiration for the design and synthesis of the geneticallymodified golden rice, which is used as a source of vitamin A to help prevent blindness in children. Overdose of vitamin A, called hypervitaminosis A is dangerous and can be fatal. Excess vitamin A is also suspected to be linked to osteoporosis. In smokers, excess vitamin A is linked to an increased rate of lung cancer, but non-smokers have a reduced rate.

Vitamin D

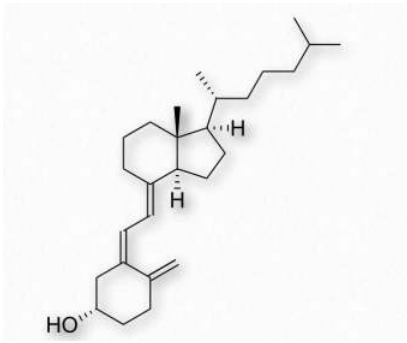


Figure 2.229 - Cholecalciferol - Vitamin D3

The active form of vitamin D plays important roles in the intestinal absorption of calcium and phosphate and thus in healthy bones. Technically, vitamin D isn't even a vitamin, as it is a compound made by the body. Rather, it behaves more like a hormone.

Derived from ultimately from cholesterol, vitamin D can be made in a reaction catalyzed by ultraviolet light. In the reaction, the intermediate 7-dehydrocholesterol is converted to cholecalciferol (vitamin D3) by the uv light (Figure 2.229). The reaction occurs most readily in the bottom two layers of the skin shown in Figure 2.230.

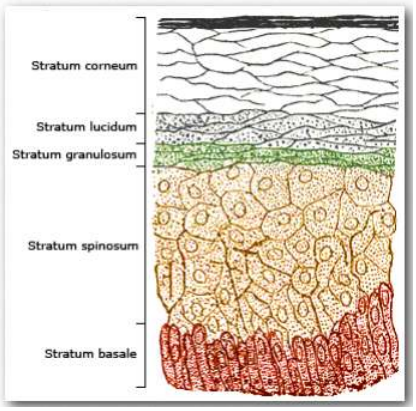


Figure 2.230 - Layers of the skin. Outside is at top.

Forms of vitamin D

Five different compounds are referred to as vitamin D. They are

Vitamin D1 - A mixture of ergocalciferol and lumisterol

Vitamin D2 - Ergocalciferol

Vitamin D3 - Cholecalciferol Vitamin

D4 - 22-Dihydroergocalciferol Vitamin

D5 - Sitocalciferol

Vitamin D3 is the most common form used in vitamin supplements and it and vitamin D2 are commonly obtained in the diet, as well. The active form of vitamin D, calcitriol (Figure 2.231), is made in the body in controlled amounts. This proceeds through two steps from cholecalciferol. First, a hydroxylation in the liver produces calcidiol and a second hydroxylation in the kidney produces calcitriol. Monocyte macrophages can also synthesize vitamin D and they use is as a cytokine to stimulate the innate immune system.

Mechanism of action

Calcitriol moves in the body bound to a vitamin D binding protein, which delivers it to target organs. Calcitriol inside of cells acts by binding a vitamin D receptor (VDR), which results in most of the vitamin's physiological effects. After binding calcitriol, the VDR migrates to the nucleus where it acts as a transcription factor to control levels of expression of calcium transport proteins (for

example) in the intestine. Most tissues respond to VDR bound to calcitriol and the result is moderation of calcium and phosphate levels in cells.

Deficiency/excess

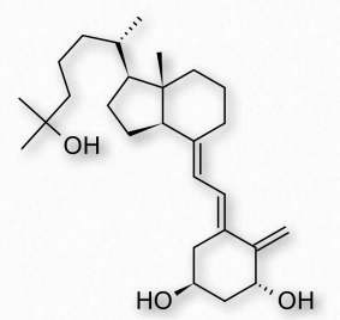


Figure 2.231 - Calcitriol - Active form of vitamin D

Deficiency of vitamin D is a cause of the disease known as rickets, which is characterized by soft, weak bones and most commonly is found in children. It is not common in the developed world, but elsewhere is of increasing concern.

Excess of vitamin D is rare, but has toxic effects, including hypercalcemia, which results in painful calcium deposits in major organs. Indications of vitamin D toxicity are increased urination and thirst. Vitamin D toxicity can lead to mental retardation and many other serious health problems.

Vitamin E

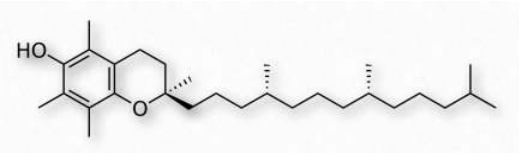


Figure 2.232 α-tocopherol - The most biologically active form of vitamin E Figure

Vitamin E comprises a group of two compounds (tocopherols and tocotrienols - Figure 2.232) and stereoisomers of each. It is commonly found in plant oils. The compounds act in cells as fat-soluble antioxidants. α-tocopherol (Figure 2.233), the most active form of the vitamin, works 1) through the glutathione peroxidase protective system and 2) in membranes to interrupt lipid peroxidation chain reactions. In both actions, vitamin E reduces levels of reactive oxygen species in cells.

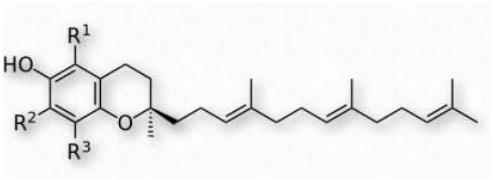


Figure 2.233 - Structure of tocotrienols

Action

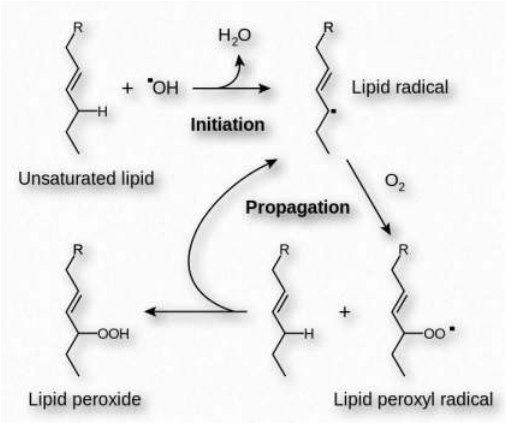


Figure 2.234 - Lipid peroxidation reactions

Vitamin E scavenges oxygen radicals (possessing unpaired electrons) by reacting with them to produce a tocopheryl radical. This vitamin E radical can be converted back to its original form by a hydrogen donor. Vitamin C is one such donor. Acting in this way, Vitamin E helps reduce oxidation of easily oxidized compounds in the lipid peroxidation reactions (Figure 2.234).

Vitamin E also can affect enzyme activity. The compound can inhibit action of protein kinase C in smooth muscle and simultaneously activate catalysis of protein phosphatase 2A to remove phosphates, stopping smooth muscle growth.

Deficiency/excess

Deficiency of vitamin E can lead to poor conduction of nerve signals and other issues arising from nerve problems. Low levels of the vitamin may be a factor in low birth weights and premature deliveries. Deficiency, however, is rare, and not usually associated with diet.

Excess Vitamin E reduces vitamin K levels, thus reducing the ability to clot blood. Hypervitaminosis of vitamin E in conjunction with aspirin can be life threatening. At lower levels, vitamin E may serve a preventative role with respect to atherosclerosis by reducing oxidation of LDLs, a step in plaque formation.

Vitamin K

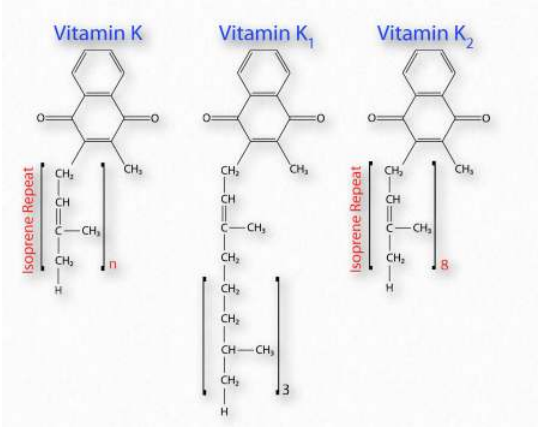


Figure 2.235 - Forms of vitamin K Image by Pehr Jacobson

Like the other fat-soluble vitamins, Vitamin K comes in multiple forms (Figure 2.235) and is stored in fat tissue in the body. There are two primary forms of the vitamin - K1 and K2 and the latter has multiple sub-forms . Vitamins K3, K4, and K5 are made synthetically, not biologically.

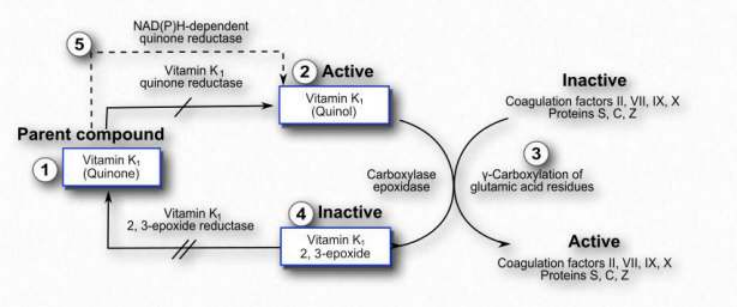


Figure 2.236 - Recycling of vitamin K Wikipedia

Action

Vitamin K is used as a co-factor for enzymes that add carboxyl groups to glutamate side chains of proteins to increase their affinity for calcium. Sixteen such proteins are known in humans. They include proteins involved in blood clotting (prothrombin (called Factor II), Factors VII, IX, and X), bone metabolism (osteocalcin, also called bone Gla protein (BGP), matrix Gla protein (MGP), and periostin) and others.

Modification of prothrombin is an important step in the process of blood clotting (see HERE). Reduced levels of vitamin K result in less blood clotting, a phenomenon sometimes referred to as blood thinning. Drugs that block recycling of vitamin K (Figure 2.236) by inhibiting the vitamin K epoxide reductase, produce lower levels of the vitamin and are employed in treatments for people prone to excessive clotting. Warfarin (coumadin) is one such compound that acts in this way and is used therapeutically. Individuals respond to the drug differentially, requiring them to periodically be tested for levels of clotting they possess, lest too much or too little occur.

Sources

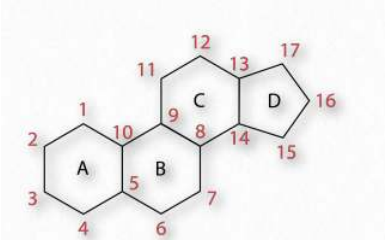


Figure 2.237 - Steroid numbering scheme Image by Pehr Jacobson

Vitamin K1 is a stereoisomer of the plant photosystem I electron receptor known as phylloquinone and is found abundantly in green, leafy vegetables. Phylloquinone is one source of vitamin K, but the compound binds tightly to thylakoid membranes and tends to have low bioavailability. Vitamin K2 is produced by microbes in the gut and is a primary source of the vitamin. Infants in the first few days before they establish their gut flora and people taking broad spectrum antibiotics may have reduced levels, as a result. Dietary deficiency is rare in the absence of damage to the small bowel. Others at risk of deficiency include people with chronic kidney disease and anyone suffering from a vitamin D deficiency. Deficiencies produce symptoms of easy bruising, heavy menstrual bleeding, anemia, and nosebleeds.

Other lipids

Cannabinoids

Cannabinoids are a group of chemicals that bind to and have effects on brain receptors (cannabinoid receptors), repressing neurotransmitter release. Classes of these compounds include endocannabinoids (made in the body), phytocannabinoids (made in plants, such as marijuana), and synthetic cannabinoids (man-made).

Endocannabinoids are natural molecules derived from arachidonic acid. Cannabinoid receptors are very abundant, comprising the largest number of G-protein- 247 Figure 2.243 - Tetrahydrocannabinol - Active ingredient in marijuana coupled receptors found in brain. The best known phytocannabinoid is Δ-9- tetrahydrocannabinol (THC), the primary psychoactive ingredient (of the 85 cannabinoids) of marijuana (Figure 2.243).

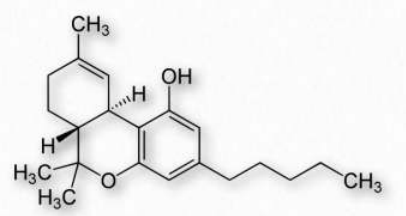


Figure 2.243 - Tetrahydrocannabinol - Active ingredient in marijuana

Anandamide

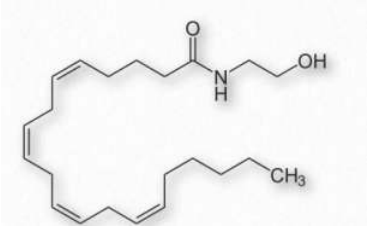


Figure 2.244 - Anandamide - An endocannabinoid

Anandamide (N-arachidonoylethanolamine - Figure 2.244) is an endocannabinoid neurotransmitter derived from arachidonic acid. It exerts its actions primarily through the CB1 and CB2 cannabinoid receptors, the same ones bound by the active ingredient in marijuana, Δ9-tetrahydrocannabinol. Anandamide has roles in stimulating eating/appetite and affecting motivation and pleasure. It has been proposed to play a role in "runners high," an analgesic effect experienced from exertion, especially among runners. Anandamide appears to impair memory function in rats.

Anandamide has been found in chocolate and two compounds that mimic its effects (N-oleoylethanolamine and Nlinoleoylethanolamine) are present as well. The enzyme fatty acid amide hydrolase (FAAH) breaks down anandamide into free arachidonic acid and ethanolamine.

Lipoxins

Lipoxins (Figure 2.245) are eicosanoid compounds involved in modulating immune responses and they have anti-inflammatory effects. When lipoxins appear in inflammation it begins the end of the process. Lipoxins act to attract macrophages to apoptotic cells at the site of inflammation and they are engulfed. Lipoxins further act to start the resolution phase of the inflammation process.

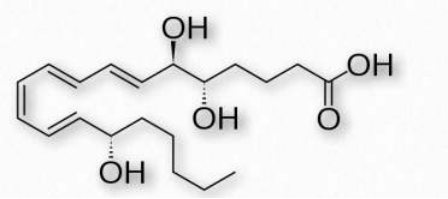


Figure 2.245 - Lipoxin A4

At least one lipoxin (aspirin-triggered LX4) has its synthesis stimulated by aspirin. This occurs as a byproduct of aspirin's acetylation of COX-2. When this occurs, the enzyme's catalytic activity is re-directed to synthesis of 15R-hydroxyeicosatetraenoic acid (HETE) instead of prostaglandins. 15R-HETE is a procursor of 15-epimer lipoxins, including aspirin-triggered LX4.

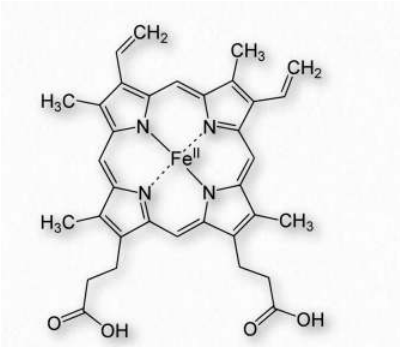
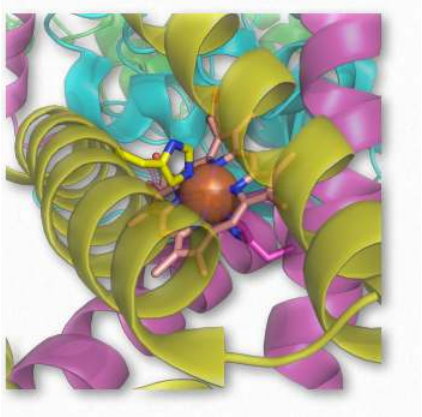


Figure 2.246 - Structure of heme B

Heme

Heme groups are a collection of protein/ enzyme cofactors containing a large heterocyclic aromatic ring known as a porphyrin ring with a ferrous (Fe++) ion in the middle. An example porphyrin ring with an iron (found in Heme B of hemoglobin), is shown in Figure 2.246. When contained in a protein, these are known collectively as hemoproteins (Figure 2.247).

Heme, of course, is a primary component of hemoglobin, but it is also found in other proteins, such as myoglobin, cytochromes, and the enzymes catalase and succinate dehydrogenase. Hemoproteins function in oxygen transport, catalysis, and electron transport. Heme is synthesized in the liver and bone marrow in a pathway that is conserved across a wide range of biology.


Figure 2.247 - Heme embedded in the succinate dehydrogenase hemoprotein Wikipedia

Porphobilinogen

Porphobilinogen (Figure 2.248) is a pyrrole molecule involved in porphyrin metabolism. It is produced from aminolevulinate by action of the enzyme known as ALA dehydratase. Porphobilinogen is acted upon by the enzyme porphobilinogen deaminase. Deficiency of the latter enzyme (and others in porphyrin metabolism) can result in a condition known as porphyria, which results in accumulation of porphobilinogen in the cytoplasm of cells.

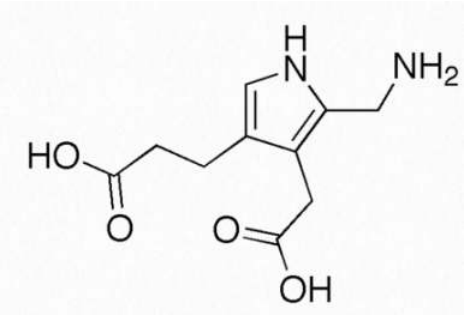


Figure 2.248 - Porphobilinogen

The disease can manifest itself with acute abdominal pain and numerous psychiatric issues. Both Vincent van Gogh and King ` George III are suspected to have suffered from porphyria, perhaps causing the “madness of King George III.” Porphyria is also considered by some to be the impetus for the legend of vampires seeking blood from victims, since the color of the skin in non-acute forms of the disease can be miscolored, leading some to perceive that as a deficiency of hemoglobin and hence the “thirst” for blood imagined for vampires.

Dolichols

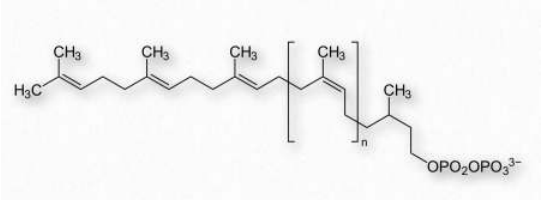


Figure 2.249 - Structure of dolichol pyrophosphate

Dolichol is a name for a group of non-polar molecules made by combining isoprene units together. Phosphorylated forms of dolichols play central roles in the N-glycosylation of proteins. This process, which occurs in the endoplasmic reticulum of eukaryotic cells, begins with a membrane-embedded dolichol pyrophosphate (Figure 2.249) to which an oligosaccharide is attached (also see HERE). This oligosaccharide contains three molecules of glucose, nine molecules of mannose and two molecules of N-acetylglucosamine.

Interestingly, the sugars are attached to the dolichol pyrophosphate with the pyrophosphate pointing outwards (away from) the endoplasmic reticulum, but after attachment, the dolichol complex flips so that the sugar portion is situated on the inside of the endoplasmic reticulum. There, the entire sugar complex is transferred to the amide of an asparagine side chain of a target protein.

The only asparagine side chains to which the attachment can be made are in proteins where the sequences Asn-X-Ser or Asn-X-Thr occur. Sugars can be removed/added after the transfer to the protein. Levels of dolichol in the human brain increase with age, but in neurodegenerative diseases, they decrease.

Terpenes



Figure 2.250 - Pine tree resin - A source of terpenes Wikipedia

Terpenes are members of a class of nonpolar molecules made from isoprene units. Terpenes are produced primarily by plants and by some insects. Terpenoids are a related group of molecules that contain functional groups lacking in terpenes.

Terpenes have a variety of functions. In plants, they often play a defensive role protecting from insects. The name of terpene comes from turpentine, which has an odor like some of the terpenes. Terpenes are common components of plant resins (think pine) and they are widely used in medicines and as fragrances. Hops, for example, gain some of their distinctive aroma and flavor from terpenes. Not all terpenes, however have significant odor.

Synthesis

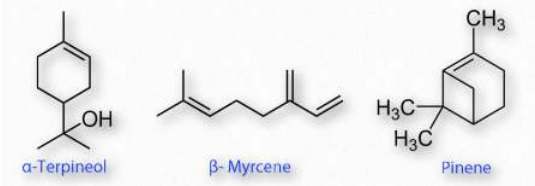


Figure 2.251 - Three monoterpenes

Terpenes, like steroids, are synthesized starting with simple building blocks known as isoprenes. There are two of them - dimethylallyl pyrophosphate and the related isopentenyl pyrophosphate and (Figures 2.252 and 2.253) which combine 1-2 units at a time to make higher order structures. Terpene synthesis overlaps and includes steroid synthesis.

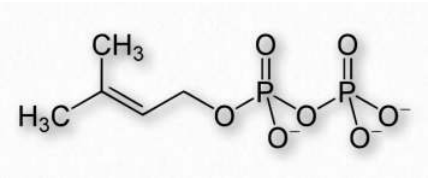


Figure 2.252 - Dimethylallyl pyrophosphate

Terpenes and terpenoids are classified according to how many isoprene units they contain. They include hemiterpenes (one unit), monoterpenes (two units), sesquiterpenes (three units), diterpenes (four units), sesterterpenes (five units), triterpenes (six units), sesquarterpenes (seven units), tetraterpenes (eight units), polyterpenes (many units). Another class of terpene-containing molecules, the norisoterpenoids arise from peroxidase-catalyzed reactions on terpene molecules.

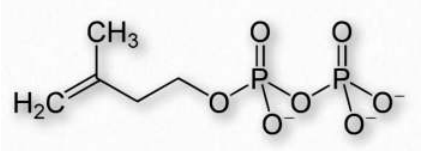


Figure 2.253 - Isopentenyl pyrophosphate

Examples

Common terpenes include monoterpenes of terpineol (lilacs), limonene (citrus), myrcene (hops), linalool (lavender), and pinene (pine). Higher order terpenes include taxadiene (diterpene precursor of taxol), lycopene (tetraterpenes), carotenes (tetraterpenes), and natural rubber (polyterpenes).

Steroid precursors geranyl pyrophosphate (monoterpene derivative), farnesyl pyrophosphate (sesquiterpene derivative), and squalene (triterpene) are all terpenes or derivatives of them. Vitamin A and phytol are derived from diterpenes.

Caffeine

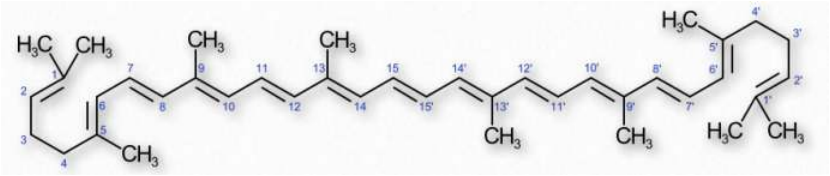


Figure 2.254 - Lycopene - A tetraterpene

Caffeine is the world's most actively consumed psychoactive drug (Figure 2.255). A methylxanthine alkaloid, caffeine is closely related to adenine and guanine and this is responsible for many effects on the body. Caffeine blocks the binding of adenosine on its receptor and consequently prevents the onset of drowsiness induced by adenosine. Caffeine readily crosses the blood-brain barrier and stimulates release of neurotransmitters. Caffeine stimulates portions of the autonomic nervous system and inhibits the activity of phosphodiesterase. The latter has the result of raising cAMP levels in cells, which activates protein kinase A and activates glycogen breakdown, inhibits TNF-α and leukotriene synthesis, which results in reduction of inflammation and innate immunity.

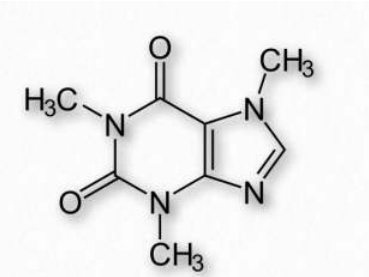


Figure 2.255 - Caffeine

Caffeine also has effects on the cholinergic system (acetylcholinesterase inhibitor), is an inositol triphosphate receptor 1 antagonist, and is a voltage independent activator of ryanodin receptors (a group of calcium channels found in skeletal muscle, smooth muscle, and heart muscle cells).

The half-life of caffeine in the body varies considerably. In healthy adults, it has a half-life of about 3-7 hours. Nicotine decreases the half-life and contraceptives and pregnancy can double it. The liver metabolizes caffeine, so the health of the liver is a factor in the halflife. CYP1A2 of the cytochrome P450 oxidase enzyme is primarily responsible. Caffeine is a natural pesticide in plants, paralyzing predator bugs.

This page titled 5.2.1: 5.2.1 Optional- other interesting lipids is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **2.8: Structure and Function - Lipids and Membranes** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

5.2.1.1: Bonus- other interesting lipids

5.2.1.1: Bonus- other interesting lipids is shared under a not declared license and was authored, remixed, and/or curated by LibreTexts.

5.3: Basic Concepts in Membranes

Source: BiochemFFA_3_1.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

Lipid bilayers

The protective membrane around cells contains many components, including cholesterol, proteins, glycolipids, glycerophospholipids, and sphingolipids. The last two of these will, when mixed vigorously with water, spontaneously form what is called a lipid bilayer (Figure 3.1), which serves as a protective boundary for the cell that is largely impermeable to the movement of most materials across it. With the notable exceptions of water, carbon dioxide, carbon monoxide, and oxygen, most polar/ionic require transport proteins to help them to efficiently navigate across the bilayer. The orderly movement of these compounds is critical for the cell to be able to 1) get food for energy; 2) export materials; 3) maintain osmotic balance; 4) create gradients for secondary transport; 5) provide electromotive force for nerve signaling; and 6) store energy in electrochemical gradients for ATP production (oxidative phosphorylation or photosynthesis). In some cases, energy is required to move the substances (active transport).

Facilitated Diffusion

In other cases, no external energy is required and they move by diffusion through specific cellular channels. This is referred to as facilitated diffusion. Before we discuss movement of materials across membranes, it is appropriate we discuss the composition of cellular membranes. Plasma membranes differ from cell walls both in the materials comprising them and in their flexibility. Cell walls will be covered near the end of this chapter.

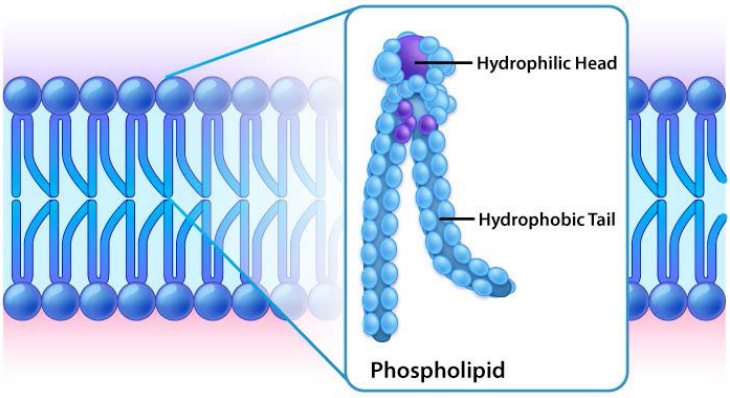


Figure 3.1: Lipid bilayer closeup Image by Aleia Kim

Though some cells do not have cell walls (animal cells) and others do (bacteria, fungi, and plants), there is commonality among cells in that they all possess plasma membranes. There is also commonality in the components of the membranes, though the relative amount of constituents varies. Figures 3.1 and 3.2 illustrate the structure and environments of plasma membranes. All plasma membranes contain a significant amount of amphiphilic substances linked to fatty acids. These include the glycerophospholipids and the sphingolipids. The fatty acid(s) are labeled as hydrophobic tails in the figures.

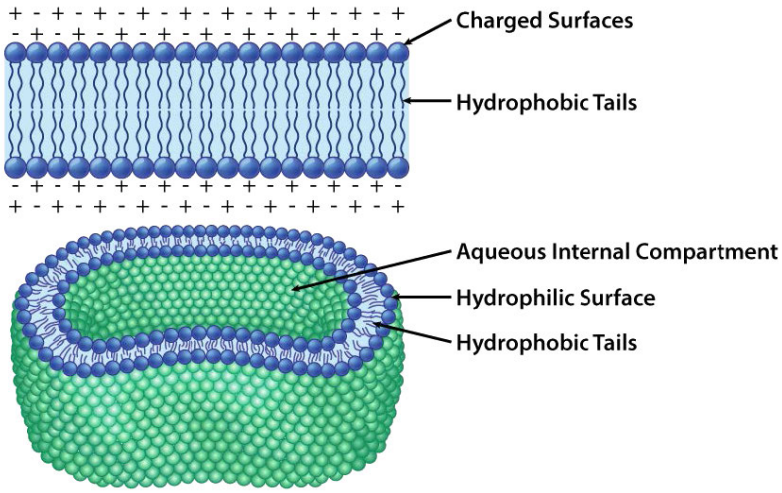


Figure 3.2: Organization of the lipid bilayer Image by Aleia Kim

Hydrophilic heads

The composition of the hydrophilic heads varies considerably. In glycerophospholipids, a phosphate is always present, of course, and it is often esterified to another substance to make a phosphatide (Figure 3.3). Common compounds linked to the phosphate (at the X position) include serine, ethanolamine, and choline. These vary in the their charges so in this way, the charge on the external or internal surface can be controlled. Cells tend to have more negative charges on the exterior half of the lipid bilayer (called the outer leaflet) and more positive charges on the interior half (inner leaflet).

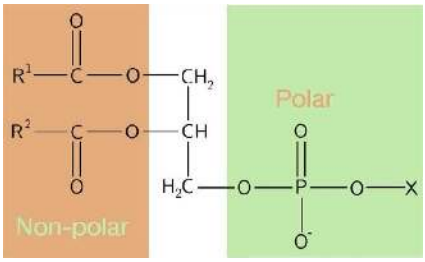


Figure 3.3 - Schematic diagram of a phosphatide

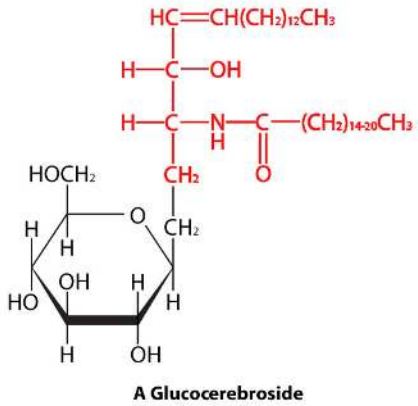


Figure 3.4: A sphingolipid. Polar head in black. Nonpolar tail in red Image by Aleia Kim

Sphingolipids

In sphingolipids (Figure 3.4), the hydrophilic head can contain a phosphate linked to ethanolamine or choline and this describes the structure of sphingomyelin, an important component of neural membranes. Most sphingolipids lack the phosphate and have instead a hydrophilic head of a single sugar (cerebrosides) or a complex oligosaccharide (gangliosides).

Water exclusion

In each case, the glycerophospholipid or sphingolipid has one end that is polar and one end that is non-polar. As we saw in the organization of amino acids with hydrophobic side chains occurring preferentially on the inside of a folded protein to exclude water, so too do the non-polar portions of these amphiphilic molecules arrange themselves so as to exclude water. Remember that the cytoplasm of a cell is mostly water and the exterior of the cell is usually bathed in an aqueous layer. It therefore makes perfect sense that the polar portions of the membrane molecules arrange themselves as they do - polar parts outside interacting with water and non-polar parts in the middle of the bilayer avoiding/excluding water.

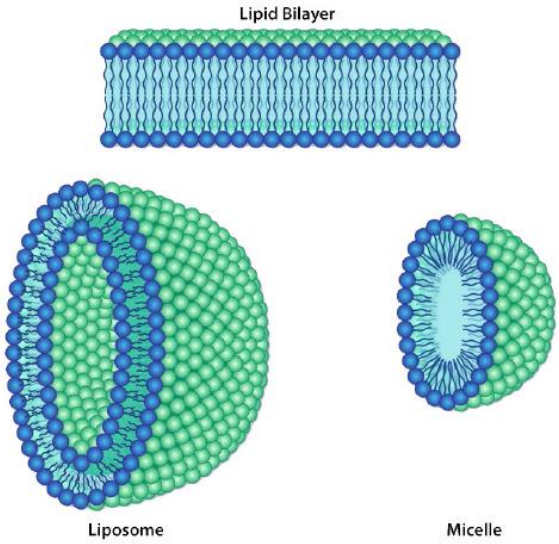


Figure 3.6 - Different lipid bilayer structures Image by Aleia Kim

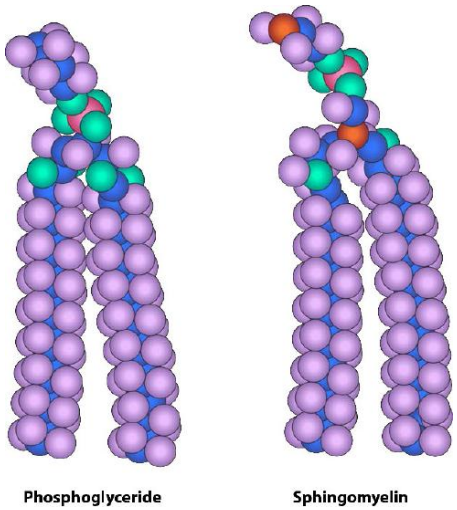


Figure 3.5 - Similarity of form between a phosphoglyceride and sphingomyelin Image by Aleia Kim

Composition Bias

The plasma membrane has distinct biases of composition relative to its inside and the outside (Figure 3.7). First, glycosylation (of lipids and proteins) has the sugar groups located almost exclusively on the outside of the cell, away from the cytoplasm (Figure 3.8). Among the membrane lipids, sphingolipids are much more commonly glycosylated than glycerophospholipids. In addition, some of the glyerophospholipids are found preferentially on one side or the other (Figure 3.7). Phosphatidylserine and phosphatidylethanolamine are found preferentially within the inner leaflet of the plasma membrane, whereas phosphatidylcholine tends to be located on the outer leaflet. In the process of apoptosis, the phosphatidylserines appear on the outer leaflet where they serve as a signal to macrophages to bind and destroy the cell. Sphingolipids are found preferentially in the plasma membrane and are almost completely absent from mitochondrial and endoplasmic reticulum membranes (Figure 3.9).

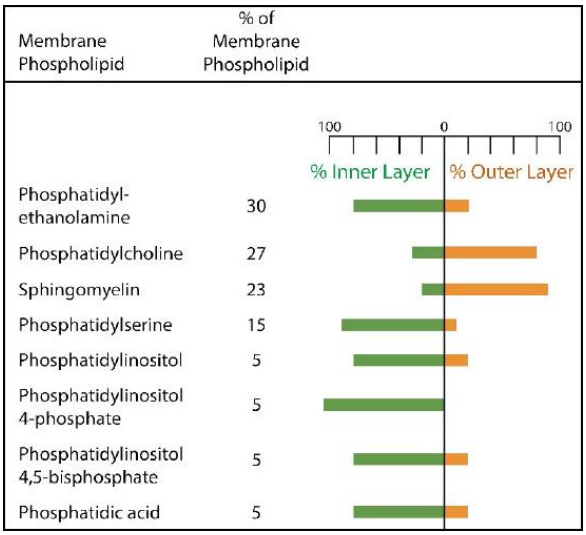


Figure 3.7: Differential distribution of membrane lipids by inner and outer leaflet Image by Pehr Jacobson

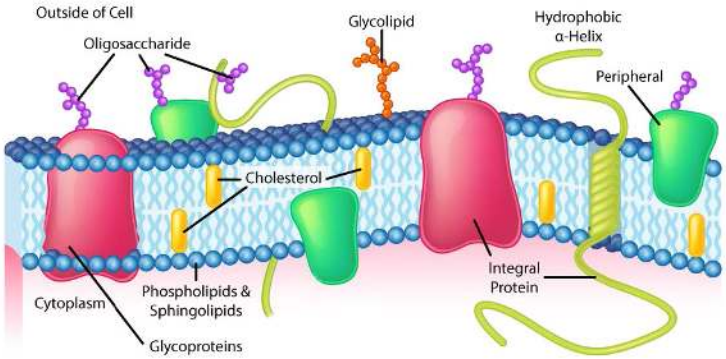


Figure 3.8 - Molecular organization of the lipid bilayer Image by Aleia Kim

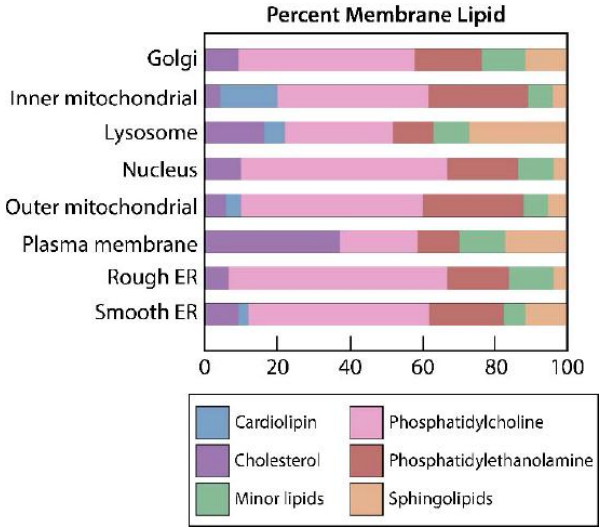


Figure 3.9 - Distribution of lipids in organelle membranes Image by Pehr Jacobson

Other components of lipid bilayer

Besides glycerophospholipids and sphingolipids, there are other materials commonly found in lipid bilayers of cellular membranes. Two important prominent ones are cholesterol (Figure 3.13) and proteins. Besides serving as a metabolic precursor of steroid

hormones and the bile acids, cholesterol's main role in cells is in the membranes. The flatness and hydrophobicity of the sterol rings allow cholesterol to interact with the nonpolar portions of the lipid bilayer while the hydroxyl group on the end can interact with the hydrophilic part.

Membrane fluidity

Cholesterol's function in the lipid bilayer is complex (Figure 3.13). It influences membrane fluidity. Figure 3.14 shows the phase transition for a membrane as it is heated, moving from a more "frozen" character to that of a more "fluid" one as the temperature rises. The mid-point of this transition, referred to as the Tm, is influenced by the fatty acid composition of the lipid bilayer compounds. Longer and more saturated fatty acids will favor higher Tm values, whereas unsaturation and short fatty acids will favor lower Tm values. It is for this reason that fish, which live in cool environments, have a higher level of unsaturated fatty acids in them - to use to make membrane lipids that will remain fluid at ocean temperatures. Interestingly, cholesterol does not change the Tm value, but instead widens the transition range between frozen and fluid forms of the membrane, allowing it to have a wider range of fluidity.

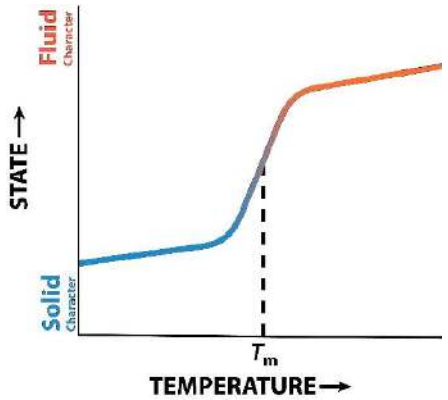


Figure 3.14 - Membrane transition temperature Image by Aleia Kim

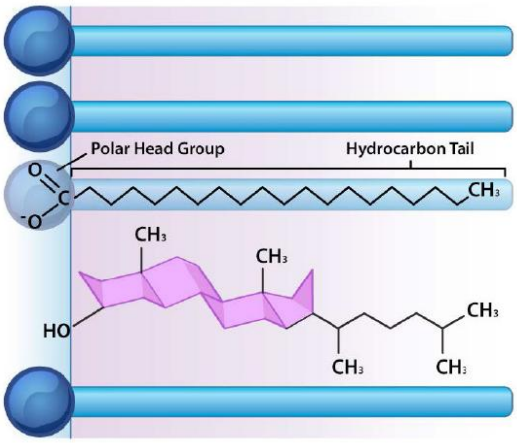


Figure 3.13 - Cholesterol in the lipid bilayer Image by Aleia Kim

Lipid Rafts

Cholesterol is also abundantly found in membrane structures called lipid rafts. Depicted in Figure 3.15, lipid rafts are organized structures within the membrane typically containing signaling molecules and other integral membrane proteins. Lipid rafts affect membrane fluidity, neurotransmission, and trafficking of receptors and membrane proteins.

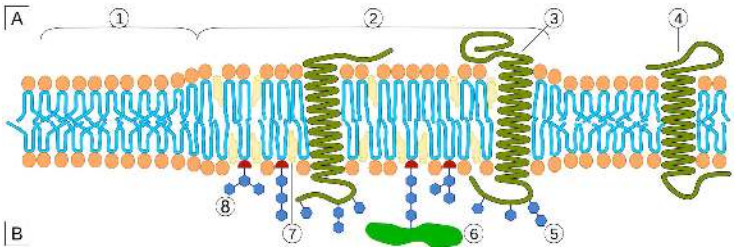


Figure 3.15 - A lipid raft - 1 = Non-raft membrane / 2 = Lipid raft / 3 = Lipid raft associated transmembrane protein / 4 = Non-raft membrane protein / 5 = Glycosylation modifications (on glycoproteins and glycolipids) / 6 = GPIanchored protein / 7 = Cholesterol / 8 = Glycolipid

Features

Distinguishing features of the rafts is that they are more ordered than the bilayers surrounding them, containing more saturated fatty acids (tighter packing and less disorganization, as a result) and up to 5 times as much cholesterol. They also are relatively rich in sphingolipids, with as much as 50% greater quantities of sphingomyelin than surrounding areas of the bilayer. The higher concentration of cholesterol in the rafts may be due to its greater ability to associate with sphingolipids (Figure 3.16). Some groups, such as prenylated proteins, like RAS, may be excluded from lipid rafts.

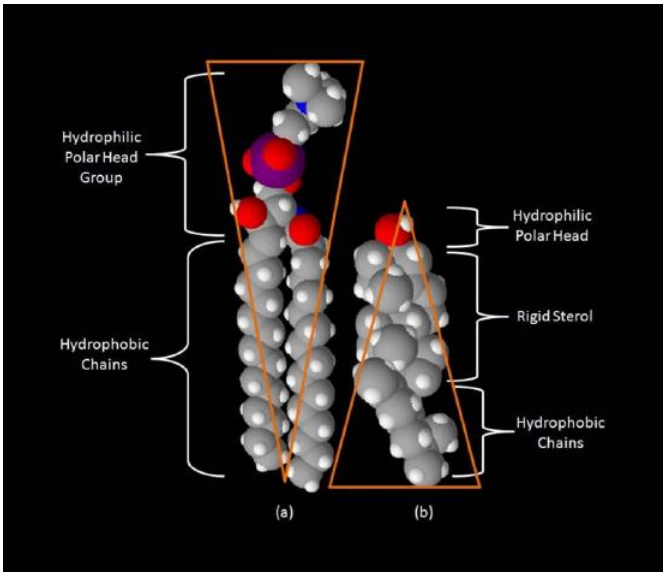


Figure 3.16 - A sphingolipid (left) associating with cholesterol (right)

Lipid rafts may provide concentrating platforms after individual protein receptors bind to ligands in signaling. After receptor activation takes place at a lipid raft, the signaling complex would provide protection from nonraft enzymes that could inactivate the signal. For example, a common feature of signaling systems is phosphorylation, so lipid rafts might provide protection against dephosphorylation by enzymes called phosphatases. Lipid rafts appear to be involved in many signal transduction processes, such as T cell antigen receptor signaling, B cell antigen receptor signaling, EGF receptor signaling, immunoglobulin E signaling, insulin receptor signaling and others. For more on signaling, see HERE.

Barrier

Transport of materials across membranes is essential for a cell to exist. The lipid bilayer is an effective barrier to the entry of most molecules and without a means of allowing food molecules to enter a cell, it would die. The primary molecules that move freely across the lipid bilayer are small, uncharged ones, such as H2O, CO2, CO, and O2, so larger molecules, like glucose, that the cell needs for energy, would be effectively excluded if there were not proteins to facilitate its movement across the membrane.

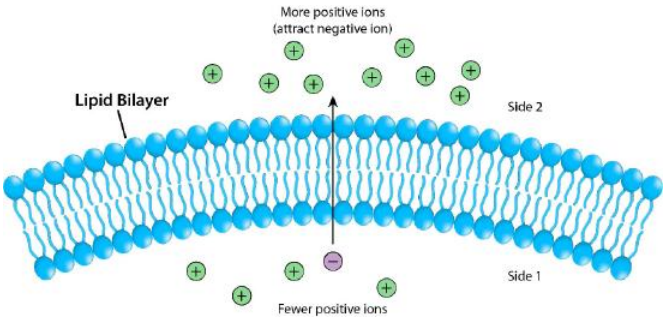


Figure 3.17 - The lipid bilayer as a barrier Image by Pehr Jacobson

Figure 3.17 depicts the barrier that the lipid bilayer provides to movement across it and the pressures (ionic attraction, in this case) that can affect movement. Potential energy from charge and concentration differences are harvested by cells for purposes that include synthesis of ATP, and moving materials against a concentration gradient in a process called active transport.

Membrane proteins

Proteins in a lipid bilayer can vary in quantity enormously, depending on the membrane. Protein content by weight of various membranes typically ranges between 30 and 75% by weight. Some mitochondrial membranes can have up to 90% protein. Proteins linked to and associated with membranes come in several types.

Transmembrane proteins

Transmembrane proteins are integral membrane proteins that completely span from one side of a biological membrane to the other and are firmly embedded in the membrane (Figure 3.18). Transmembrane proteins can function as docking sites for attachment (to the extracellular matrix, for example), as receptors in the cellular signaling system, or facilitate the specific transport of molecules into or out of the cell.

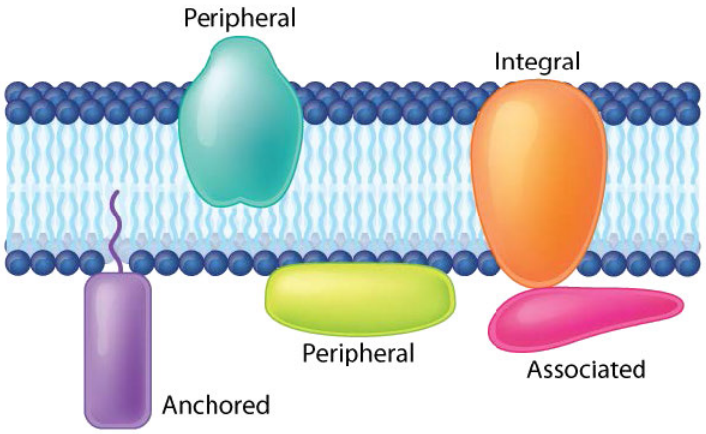


Figure 3.18 - Membrane protein types Image by Aleia Kim (Note from AG: I can't change the image, but I believe that turquoise-colored blob labeled "peripheral" should be labeled "integral" protein. If you compare orange and turquoise proteins: they are both integral, but the orange one is also transmembrane, while turquoise is not.)

Examples of integral and transmembrane proteins include those involved in transport (e.g., Na+/K+ ATPase), ion channels (e.g., potassium channel of nerve cells) and signal transduction across the lipid bilayer (e.g., G-Protein Coupled Receptors).

Peripheral membrane proteins interact with just the outer part of the bilayer (usually does not involve hydrophobic interactions), but do not project through it. A good example is phospholipase A2, which cleaves fatty acids from glycerophospholipids in membranes. Associated membrane proteins typically do not have external hydrophobic regions, so they cannot embed in a portion

of the lipid bilayer, but are found near them. Such association may arise as a result of interaction with other proteins or molecules in the lipid bilayer. A good example is enzyme ribonuclease.

Anchored membrane proteins

Anchored membrane proteins are not themselves embedded in the lipid bilayer, but instead are attached to a molecule (typically a fatty acid) that is embedded in the membrane (Figure 3.19). The oncogene family of proteins known as ras are good examples. These proteins are anchored to the lipid bilayer by attachment to non-polar farnesyl groups catalyzed by the enzyme farnesyltransferase.

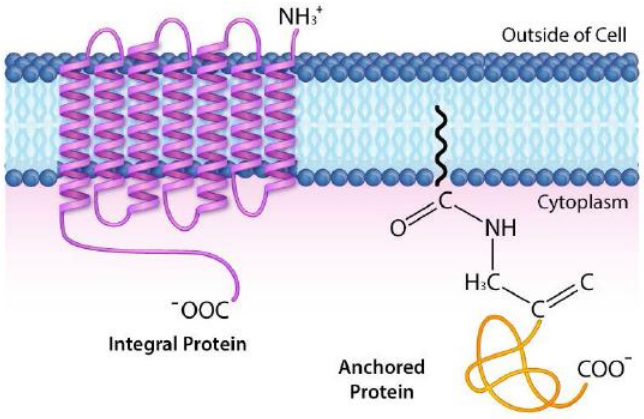


Figure 3.19 - Integral and anchored proteins Image by Aleia Kim

Blood Types

Cells have hundreds-thousands of membrane proteins and the protein composition of a membrane varies with its function and location. Glycoproteins embedded in membranes play important roles in cellular identification. Blood types, for example, differ from each other in the structure of the carbohydrate chains projecting out from the surface of the glycoprotein in their membranes (Figure 3.21).

	Group A	Group B	Group AB	Group O
Red blood cell type	A	B	AB	O
Antibodies in Plasma	Anti-B	Anti-A	None	Anti-A and Anti-B
Antigens in Red Blood Cell	A antigen	B antigen	A and B antigens	None

Figure 3.21

Optional review from biology and general chemistry: Osmotic Pressure

Membranes provide barriers/boundaries for most molecules, but the permeability of water across a lipid bilayer creates a variable that must be considered. The variable here is osmotic pressure. Osmotic pressure (loosely) refers to the tendency of a solution to take in water by the process of osmosis. In Figure 3.22, one can see a visual representation of the concept of the pressure.

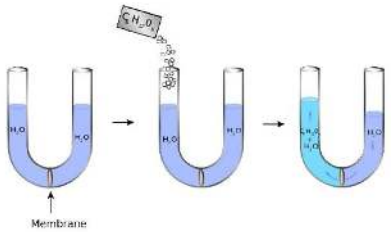


Figure 3.22

A U-shaped tube has at its bottom a semipermeable membrane. Water can pass through the membrane, but sugar molecules (C6H12O6) cannot. On the left side, sugar is added creating a concentration difference between the right and left chambers. Water diffuses across the membrane from right to left in an attempt to equalize the concentrations, causing the level of the right side to decrease and the left side to increase. The pressure resulting from the differences in height is felt at the membrane.

Equalizing concentrations

The liquid on the right does not completely move to the left, though, as might be expected if the only force involved is equalizing the concentration of sugar across the membrane (no sugar on right = no water). Instead, an equilibrium of sorts of water levels is reached even though the concentrations don't equal out. The pressure existing at the membrane then from the differences in level corresponds to the osmotic pressure of the mixture. The osmotic pressure of a solution is the pressure difference needed to halt the flow of solvent across a semipermeable membrane. Osmotic pressure can also be thought of as the pressure required to counter osmosis. The osmotic pres- Figure 3.21 - Blood types arise from cell surface glycoproteins Figure 3.22 - Osmotic pressure. Water diffuses leftwards to try to equalize the solute concentration. The pressure realized at the membrane in the right figure is the osmotic pressure sure of a dilute solution mathematically behaves like the ideal gas law

$$P_{osmotic} = \frac{nRT}{V} \tag{5.3.1}$$

where n is the number of moles, R is the gas constant, T is the temperature in Kelvin, and V is the volume.

It is more convenient in solutions to work with molarity, so

$$P_{osmotic} = MR^*T \tag{5.3.2}$$

where M is the molarity of the dissolved molecules, R* is the gas constant expressed in (L atm)/(K mol), and T is the temperature. The Greek letter Π is used to refer to the $P_{osmotic}$term, so

$$\Pi = MR^*T \tag{5.3.3}$$

Remember when calculating the molarity to include the molarity of each particle. For example, when one dissolves sucrose in solution, it does not split into smaller particles, so

$$Molarity_{Particles} = Molarity_{Sucrose} \tag{5.3.4}$$

However, for salts, like KOH, which forms two ions in solution (K+ and OH-),

$Molarity_{Particles}$= 2* $Molarity_{KOH}$.

Significant consideration

Osmotic pressure is a significant consideration for cells. Consider the fact that water can move freely across cellular membranes, but most of the contents of the cell, such as proteins, DNA, ions, sugars, etc., cannot. Second, the concentration of these compounds inside the cell is different than the concentration of them outside of the cell. Third, since water can move through the lipid bilayer, it will tend to move in the direction that will tend to equalize solute.

Hypotonic, hypertonic, isotonic

We consider three situations (Figure 3.23). First, if the concentration of solutes is greater inside the cell than outside, water will tend to move into the cell, causing the cell to swell. This circumstance is called hypotonic. Conversely, if the solute concentration is greater outside the cell than inside of it, water will exit the cell and the cell with shrink. This is a hypertonic situation. Last, if the concentrations of solutes into and outside of the cell is equal, this is called an isotonic solution. Here, no movement of water occurs across the cell membrane and the cell retains its size.

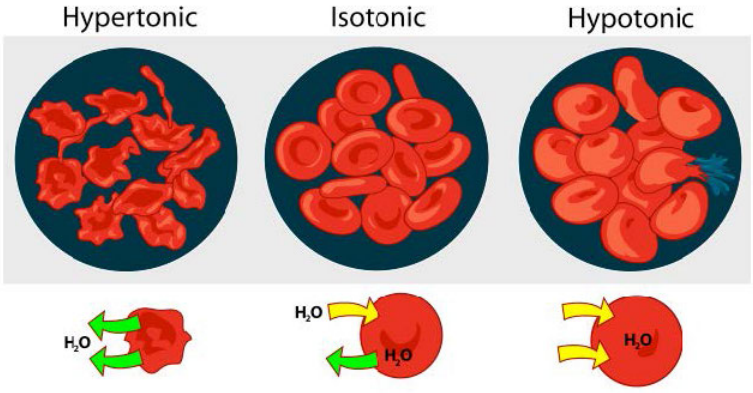
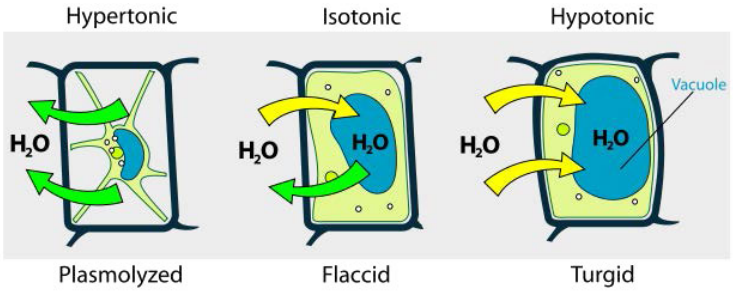


Figure 3.23 - The effect of three different osmotic conditions on red blood cells concentrations on either side of the membrane

If the osmotic pressure is greater than the forces holding together a cellular membrane, the cell will rupture. Because of this, some cells have built in defenses to prevent problems. Plant cells, for example have a fairly rigid cell wall that resists expansion in hypotonic solutions (Figure 3.24). Bacteria also have a cell wall that provides protection.



This page titled 5.3: Basic Concepts in Membranes is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **3.1: Basic Concepts in Membranes** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

5.4: Transport Across Membranes

Source: BiochemFFA_3_2.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

Movement of materials across membranes

As noted earlier, it is essential for cells to be able to uptake nutrients. This function along with movement of ions and other substances is provided by proteins/protein complexes that are highly specific for the compounds they move.

Selective movement of ions by membrane proteins and the ions' extremely low permeability across the lipid bilayer are important for helping to maintain the osmotic balance of the cell and also for providing for the most important mechanism for it to make ATP - the process of oxidative phosphorylation.

Terminology

A protein involved in moving only one molecule across a membrane is called a uniport (Figure 3.25). Proteins that move two molecules in the same direction across the membrane are called symports (also called synporters, synports, or symporters). If two molecules are moved in opposite directions across the bilayer, the protein is called an antiport. Proteins involved in moving ions are called ionophores.

If the action of a protein in moving ions across a membrane results in a net change in charge, the protein is described as electrogenic and if there is no change in charge the protein is described as electroneutral (Figure 3.26). When the driving force for movement through the membrane protein is simply diffusion, the process is called facilitated diffusion or passive transport and when the process requires other energy input, the process is called active transport.

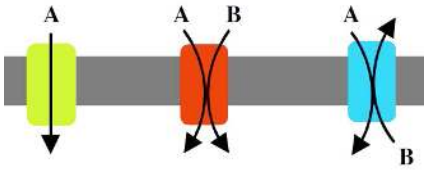


Figure 3.25 - A uniport, a symport, and an antiport

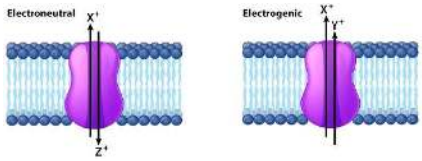


Figure 3.26 - Electroneutral and electrogenic transporters - Image by Aleia Kim

Channels and transporters

With respect to movement of materials through membrane proteins, there is a difference between channels (sometimes called pores) and transporters. Channels largely provide openings with some specificity and molecules pass through them at close to the rate of diffusion. They usually involve movement of water or ions. Examples would be the sodium or potassium channels of nerve cells. Transporters have high specificity and transfer rates that are orders of magnitude slower. Transport proteins include the sodium-potassium pump, the sodium-calcium exchanger, and lactose permease, amongst many others).

Facilitated diffusion

As noted, the driving force for facilitated diffusion is concentration, meaning that in facilitated diffusion, materials will only move from a higher concentration to a lower concentration and that at the end of the process, the concentration of materials on each side of a bilayer will be equal (Figure 3.28). This may work well in many cases.

For example, the blood concentration of glucose is sufficiently high that red blood cells can use facilitated diffusion as a means of acquiring glucose. Other cells, further removed from the blood supply where the glucose concentration is lower, must use active transport mechanisms because there is not a sufficient concentration of glucose to provide cells with the glucose they need.

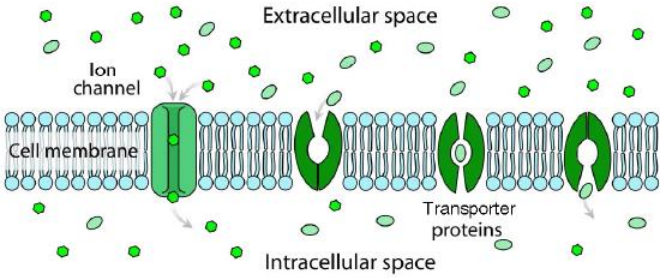


Figure 3.27 - Ion channel and transporter proteins

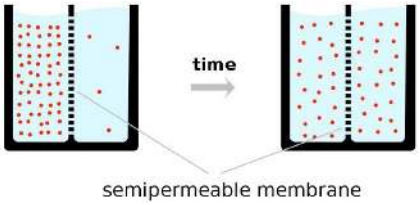


Figure 3.28 - In diffusion, solutes move from high concentration to lower concentration and eventually equalize

Ion channels

Ion channels are pore-forming membrane proteins in the membranes of all cells that regulate movement of selected ions across a membrane (Figures 3.29 & 3.30). They help to establish the resting membrane potential and to affect action potentials and other electrical signals. They are very important in the process of nerve transmission. Ion channels control the flow of ions across secretory and epithelial cells, and consequently help to regulate cell volume by affecting osmotic pressure.

Ion channels are essential features of almost all cells, functioning as selective “tunnels” that restrict movement through them to ions with specific characteristics (typically size). The size of the opening is very narrow (usually one or two atoms wide) and is able to select even against ions that are too small.

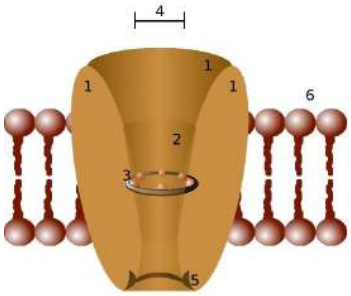


Figure 3.29 - Schematic structure of an ion channel protein - 1 = General structure / 2 = Entrance / 3 = Selectivity filter / 4 = Effective diameter / 5 = Controllable opening (Note from AG: what does not seem right about this picture?)

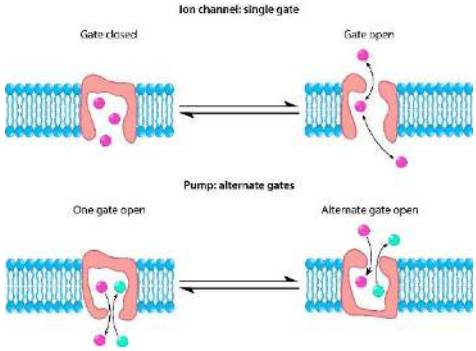


Figure 3.30 - Two types of ion channel - single and double gates - Image by Pehr Jacobson

Control mechanisms

Ion channels are controlled by mechanisms that include voltage, ligands, light, temperature, and mechanical deformation (stretch activated). Ligand-gated ion channels (LGICs) are transmembrane proteins which open to selectively allow ions such as Na^+, K^+, Ca^{++}, or Cl^- to pass through the membrane in response to the binding of a ligand messenger.

Sound waves cause mechanical deformation of hair cells in the ear. This results in the opening of ion channels and initiation of a nerve signal to the brain.

Sodium ion channels in the tongue for sugar receptors open in response to binding of sucrose, allowing sodium concentration in the nerve cell to increase and initiate a nerve signal to the brain. In this case, the default for the gate is to be closed and it opens in response to binding of a ligand (sucrose).

In light sensing cells of the eye, calcium gates are open by default, but stimulation by light causes them to close, triggering a series of events that result in a signal being sent the brain about the perception of light. Thus, in this case, the stimulus (light) causes an open channel to close.

Moving the other direction, nerve signals originating in the brain travel to muscle tissue and through a complicated set of exchanges, result in the opening of calcium gates of muscle cells, increasing the concentration of calcium and stimulating muscular contraction (see HERE).

Voltage gated channels are essential for transmission of nerve signals, a process discussed in more depth HERE.

Ion movement through channels

The ability of ion channels to select against ions too large is intuitive - the size of the opening in the ion channel simply isn't big enough for a larger ion to fit through the opening. Potassium, for example, passes through sodium channels rarely because the opening is too small.

Potassium channels that are selective for potassium ions must be big enough to allow potassium to enter, but if size were the only selection means, then sodium ions would also readily pass through potassium channels, since sodium ions (0.95 Å) are smaller than potassium ions (1.33 Å). In order for potassium channels to select against sodium ions and favor potassium ions, other considerations come into play.

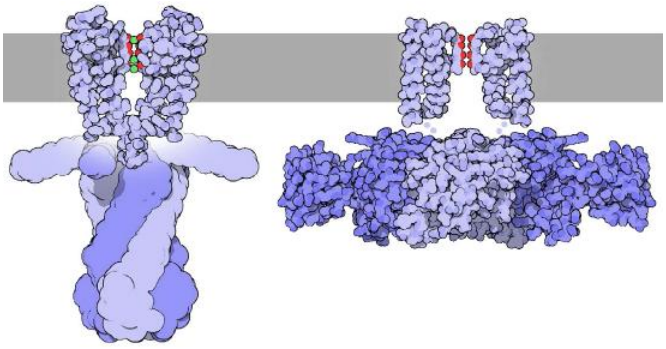


Figure 3.31 - A potassium channel closed (left) and open (right) - David Goodsell - "Molecule of the Month: Potassium Channels". (from Wikipedia)

Hydration shell

To understand this unique selectivity, it is important to understand how ions move through channels. Before an ion can pass through a channel, it must first be dissociated from (stripped of) the water molecules in its hydration shell - water molecules surrounding ions in aqueous solutions (Figure 3.32). This process requires an input of energy. The initial energy required to strip the water molecules from the hydration shell has been compared to the activation energy of an enzymatic reaction.

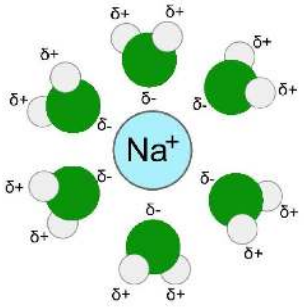


Figure 3.32 - Sodium surrounded by water molecules in a hydration shell

Comparable to enzymes

Just as enzymes lower the activation energy of enzymatic reactions and thus allow them to more readily occur, so too do channel proteins lower the energy requirements for a molecule to traverse a lipid bilayer. In the absence of the channel protein, the dehydration energy is mostly prohibitive for most polar molecules to occur, so very few make it across the lipid bilayer without the channel protein. This is why ion channel/transport proteins are so important to the cell.

After the water has been stripped, the ion can pass through the channel and when it arrives at the other side of the channel, the diffusing ion becomes rehydrated, thus regaining the energy that was required initially to strip away the water molecules from the ion.

Selectivity of the potassium channel

The potassium channel (Figure 3.33) uses the dimensions of the potassium ion precisely to shepherd it through the channel. The sodium ion, which has different dimensions has a more difficult time making it through the channel despite its smaller size. The reason this is rooted in the energy required for dehydration.

For potassium ions, after the water has been stripped off, precisely positioned carbonyl groups along the channel help to stabilize the ion as it moves. The sodium ion, on the other hand is too small and does not make efficient connections with carbonyl groups and thus has a more difficult path. Because of this, the energy difference between dehydration and rehydration of a sodium ion in a potassium channel is energetically unfavorable (requires net input of energy) but the same process for a potassium ion is energetically favorable (results in a net gain of energy).

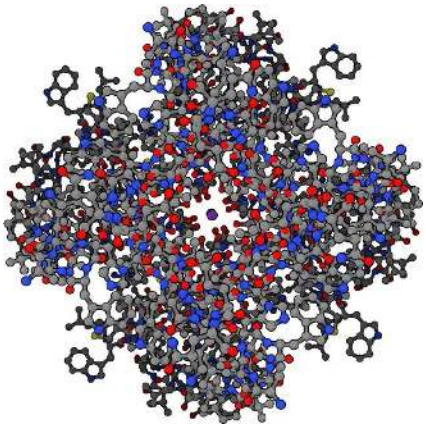
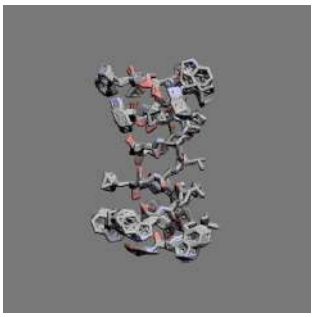


Figure 3.33 - A potassium ion (center) transiting the potassium channel - David Goodsell



Movie 3.1 - Gramicidin A Wikipedia (animated gif, download to view)

Energy factor

Thus the selection in favor of potassium and against sodium ions in a potassium channel is based on energy, not physical size, whereas in the selection of sodium ions over potassium ions in a sodium channel, size is the primary consideration.

Ion balance

The movement of ions across a lipid bilayer is tightly regulated, and with good reason. Maintaining a proper balance of ions inside and outside of cells is important for maintaining osmotic balance. It is also important inside and outside of organelles like the mitochondria and chloroplasts for energy generation. If the ionic balance of a cell is sufficiently disturbed by an uncontrolled ionophore, a cell may die.

Gramicidin

Gramicidins (Movie 3.1) are antibiotic polypeptides synthesized by the soil bacterium known as Bacillus brevis. These small pentadecapeptides (15 amino acids) are synthesized by the bacterium to kill other bacteria.

When released by the Bacillus brevis, the gramicidins insert themselves in the membranes of Gram positive bacteria and allow the movement of sodium ions into the target cells, ultimately killing them. Gramicidins can also cause hemolysis in humans so they cannot be used internally, but instead are used topically.

Aquaporins

Aquaporins are pore-containing integral membrane proteins that selectively permit passage of water molecules in and out of the cell, while preventing ions and other solutes from moving (Figures 3.34 & 3.35). Some aquaporins called aquaglyceroporins, also transport other small uncharged entities, such as glycerol, ammonia, urea, and CO2, across the membrane,. The water pores are completely impermeable to charged molecules, such as protons, which is important for the preserving the membrane's electrochemical potential difference.

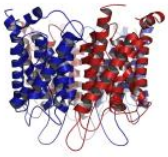


Figure 3.34 - View of aquaporin from the side - Wikipedia

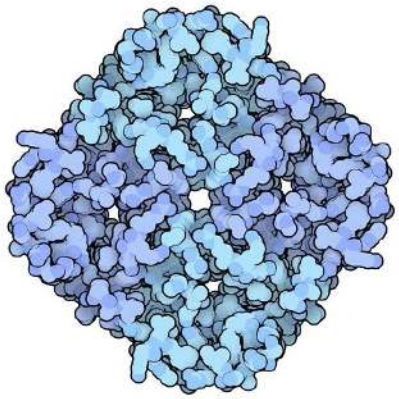


Figure 3.35 - View of aquaporin from the top - David Goodsell

Porins

Porins are proteins containing a β-barrel structure that crosses the cell membrane/wall and acts as a pore/channel through which specific molecules diffuse. Porins are found in the outer membrane of Gram-negative bacteria and some Gram-positive bacteria, mitochondria, and chloroplasts.

Porins typically transport only one group of molecules or, in some cases, one specific molecule. Antibiotics, such as β-lactam and fluoroquinolone pass through porins to reach the cytosol of Gram-negative bacteria. Bacteria may develop resistance to these antibiotics when a mutation occurs to the porin involved that results in exclusion of the antibiotics that would otherwise pass through.

Transporter proteins

Not all facilitated transport occurs through ion channel proteins. Transporter proteins, as noted earlier (HERE and Figure 3.27) facilitate movement of materials across a lipid bilayer, but are slower than ion channels. Figure 3.36 illustrates a transporter protein in action. As can be seen, transporter proteins rely on a specific receptor site for proper recognition of the molecule to be moved.

Binding of the proper molecule causes a conformational change in the shape of the protein (an eversion) which results in a flipping of the open side of the protein to the other side of the lipid bilayer. In this way, the molecule is moved. Like ion channels, transporter proteins facilitate movement of materials in either direction, driven only by the concentration difference between one side and the other.

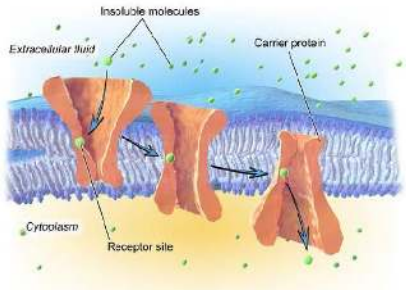


Figure 3.36 - Facilitated diffusion with a transporter protein - Wikipedia

Active transport

All of the transport mechanisms described so far are driven solely by a concentration gradient - moving from higher concentrations in the direction of lower concentrations. These movements can occur in either direction and, as noted, result in equal concentrations on either side of the bilayer, if allowed to go to completion. Many times, however, cells must move materials against a concentration gradient and when this occurs, another source of energy is required. This process is known as active transport.

A good definition of active transport is that during active transport, at least one molecule is being moved against a concentration gradient. A common, but not exclusive, energy source is ATP (see Na+/K+ ATPase), but other energy sources are also employed.

For example, the sodium-glucose transporter uses a sodium gradient as a force for actively transporting glucose into a cell. Thus, it is important to know that not all active transport uses ATP energy.

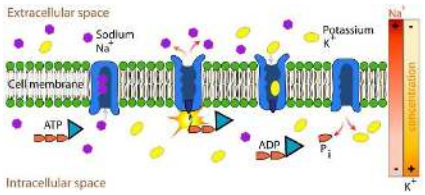


Figure 3.37 - An overview of active transport by the Na+K+ ATPase

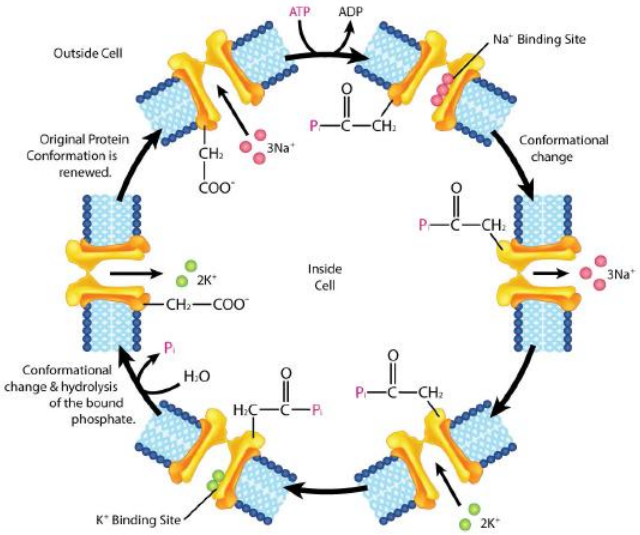


Figure 3.38 - Sequential steps in the active transport of ions by theNa+K+ ATPase - Wikipedia (Note from AG: do not worry about details of this transporter, just focus on the general idea/summary)

Na+/K+ ATPase

An important integral membrane transport protein is the Na+/K+ ATPase antiport (Figures 3.37 and 3.38), which moves three sodium ions out of the cell and two potassium ions into the cell with each cycle of action. In each case, the movement of ions is against the concentration gradient. Since three positive charges are moved out for each two positive charges moved in, the system is electrogenic.

The protein uses the energy released during ATP hydrolysis to create ion gradients that are important both in maintaining cellular osmotic pressure and (in nerve cells) for creating the sodium and potassium gradients necessary for signal transmission. Failure of the system to function results in swelling of the cell due to movement of water into the cell through osmotic pressure. The transporter expends about one fifth of the ATP energy of animal cells.

The cycle of action occurs as follows:

1. Pump binds ATP followed by binding of 3 Na+ ions from cytoplasm of cell
2. ATP hydrolysis results in phosphorylation of aspartate residue of pump. ADP is released
3. Phosphorylated pump undergoes conformational change to expose Na+ ions to exterior of cell. Na+ ions are released.
4. Pump binds 2 extracellular K+ ions.
5. Pump dephosphorylates causing it to expose K+ ions to cytoplasm as pump returns to original shape.
6. Pump binds 3 Na+ ions, binds ATP and releases 2 K+ ions to restart process

The Na+/K+ ATPase is classified as a P-type ATPase. This category of pump is notable for having a phosphorylated aspartate intermediate and is present across the biological kingdoms - bacteria, archaeans, and eukaryotes.

Na+/glucose transporter

Absorbing nutrients from the digestive system is necessary for animal life. The sodium/glucose transport protein is an electrogenic symporter that moves glucose into intestinal cells. It is found in the intestinal mucosa and the proximal tubule of the nephron of the kidney. The sodium/glucose transport system functions in the latter to promote reabsorption of glucose.

The pump works in conjunction with the Na+/K+ transport system. The gradient of sodium ions built up by the Na+/K+ pump is used as an energy source to drive movement of glucose into cells (see Figure 3.38). Use of an ion gradient established by a separate pump is known as secondary active transport. For intestinal mucosa, the pump transports glucose out of the gut and into gut cells. Later, the glucose is exported out the other side of the gut cells to the interstitial space for use in the body.

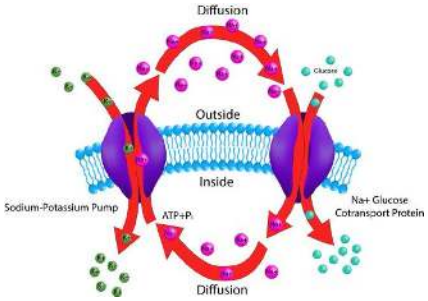


Figure 3.46 - The sodium/glucose pump (right) and the Na+/K+ ATPase (left). The sodium gradient generated by the Na+/K+ ATPase is used by the sodium/glucose pump to import glucose into the cell. Wikipedia

Not all transport proteins perform active transport.

GLUTs

GLUTs (GLUcose Transport proteins) are uniport, type III integral membrane proteins that participate in the transport of glucose across membranes into cells. GLUTs are found in all phyla and are abundant in humans, with 12 GLUT genes. GLUT1, in erythrocytes is well-studied. Through GLUT 1, glucose enters and passes through it via facilitated diffusion at a rate that is 50,000 higher than in its absence. GLUTs of various types are found in different cells of the body. The one in red blood cells is known as GLUT 1 and has 12 membrane-spanning hydrophobic helices.

Though the structure of GLUT 1 is not known, it is speculated that the 12 helices form a chamber able to form hydrophilic bonds with glucose to facilitate its passage.

GLUT 1 levels in erythrocytes go up as glucose levels decrease and decrease when glucose levels go down. GLUT 1 can also transport ascorbate (vitamin C) in addition to glucose in mammals (such as humans) that do not produce their own vitamin C.

Glut 4

GLUT 4 is regulated by insulin and is found primarily in adipose and striated muscle tissue. Insulin alters intracellular trafficking pathways in response to increases in blood sugar to favor movement of various GLUT proteins (including GLUT 4) from intracellular vesicles to the cell membrane, thus stimulating uptake of the glucose. GLUT 4 is also found in the hippocampus where, if trafficking is disrupted, the result can be depressive behavior and cognitive dysfunction.

For all of the GLUT proteins, a key to keeping the glucose in the cell is phosphorylation of it by the glycolysis enzyme, hexokinase, in the cytoplasm. Phosphorylated molecules cannot enter GLUTs and don’t have an easy means of exiting the cell.

Glucose Transporters

Name	Tissue	K_M (μM)	Comments
GLUT1	All mammalian tissues	1	Basal glucose uptake
GLUT2	Liver and pancreatic β cells	15-20	Participates in insulin regulation in the pancreas; removes excess glucose from the blood in the liver
GLUT3	All mammalian tissues	1	Basal glucose uptake
GLUT4	Muscle and fat cells	5	Concentration in plasma membrane of muscles rises with endurance training
GLUT5	Small intestine	---	Fructose transport

Figure 3.52 - GLUTs found in cells - Image by Aleia Kim

Note: while we consider each type of transport protein separately, they work together in the cell. Consider how the three transporters described above work together to get glucose you eat from your intestines into to your bloodstream.

This page titled 5.4: Transport Across Membranes is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **3.2: Transport in Membranes** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

5.4.1: Optional deep dive in to membrane transport

Source: BiochemFFA_3_2.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

Active transport

All of the transport mechanisms described so far are driven solely by a concentration gradient - moving from higher concentrations in the direction of lower concentrations. These movements can occur in either direction and, as noted, result in equal concentrations on either side of the bilayer, if allowed to go to completion. Many times, however, cells must move materials against a concentration gradient and when this occurs, another source of energy is required. This process is known as active transport.

A good definition of active transport is that during active transport, at least one molecule is being moved against a concentration gradient. A common, but not exclusive, energy source is ATP (see Na+/K+ ATPase), but other energy sources are also employed. For example, the sodium-glucose transporter uses a sodium gradient as a force for actively transporting glucose into a cell. Thus, it is important to know that not all active transport uses ATP energy.

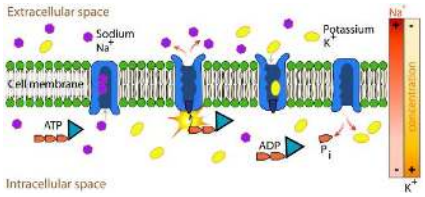


Figure 3.37 - An overview of active transport by the Na+K+ ATPase

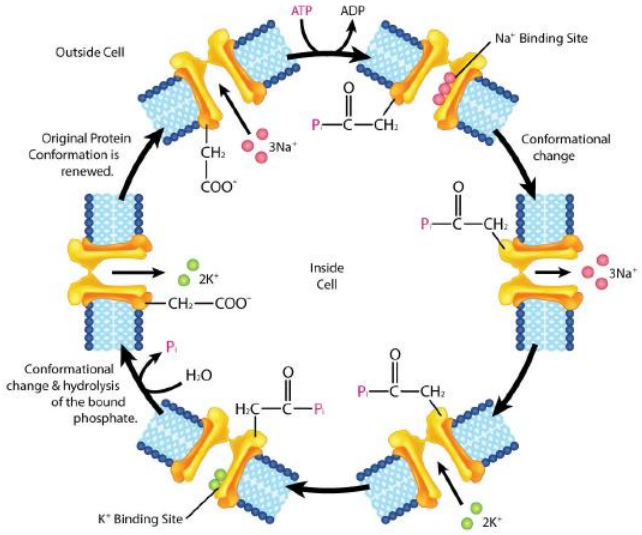


Figure 3.38 - Sequential steps in the active transport of ions by theNa+K+ ATPase - Wikipedia (Note from AG: do not worry about details of this transporter, just focus on the general idea/summary)

Na+/K+ ATPase

An important integral membrane transport protein is the Na+/K+ ATPase antiport (Figures 3.37 and 3.38), which moves three sodium ions out of the cell and two potassium ions into the cell with each cycle of action. In each case, the movement of ions is against the concentration gradient. Since three positive charges are moved out for each two positive charges moved in, the system is electrogenic.

The protein uses the energy of ATP to create ion gradients that are important both in maintaining cellular osmotic pressure and (in nerve cells) for creating the sodium and potassium gradients necessary for signal transmission. Failure of the system to function

results in swelling of the cell due to movement of water into the cell through osmotic pressure. The transporter expends about one fifth of the ATP energy of animal cells. The cycle of action occurs as follows:

1. Pump binds ATP followed by binding of 3 Na+ ions from cytoplasm of cell
2. ATP hydrolysis results in phosphorylation of aspartate residue of pump. ADP is released
3. Phosphorylated pump undergoes conformational change to expose Na+ ions to exterior of cell. Na+ ions are released.
4. Pump binds 2 extracellular K+ ions.
5. Pump dephosphorylates causing it to expose K+ ions to cytoplasm as pump returns to original shape.
6. Pump binds 3 Na+ ions, binds ATP and releases 2 K+ ions to restart process

The Na+/K+ ATPase is classified as a P-type ATPase. This category of pump is notable for having a phosphorylated aspartate intermediate and is present across the biological kingdoms - bacteria, archaeans, and eukaryotes.

ATPase types

ATPases have roles in either the synthesis or hydrolysis of ATP and come in several different forms.

- F-ATPases (F1FO-ATPases) are present in mitochondria, chloroplasts and bacterial plasma membranes and are the prime ATP synthesizers for these systems. Each uses a proton gradient as its energy source for ATP production. Complex V of the mitochondrion is an F-type ATPase.
- V-ATPases (V1VO-ATPases) are mostly found in vacuoles of eukaryotes . They utilize energy from ATP hydrolysis to transport solutes and protons into vacuoles and lysosomes, thus lowering their pH values.

The V-type and F-type ATPases are very similar in structure. The V-type (Figure 3.39) uses ATP to pump protons into vacuoles and lysosomes, whereas F-types use proton gradients of the mitochondria and chloroplasts to make ATP.

- A-ATPases (A1AO-ATPases) are found in archaeans and are similar to F-ATPases in function.
- P-ATPases (E1E2-ATPases) are in bacteria, fungi and in eukaryotic plasma membranes and organelles. They transport a diversity of ions across membranes. Each has a common mechanism of action which include autophosphorylation of a conserved aspartic acid side chain within it. Examples of P-type ATPases include the Na+/K+ ATPase and the calcium pump.
- E-ATPases are enzymes found on the cell surface. They hydrolyze a range of extracellular nucleoside triphosphates, including ATP.

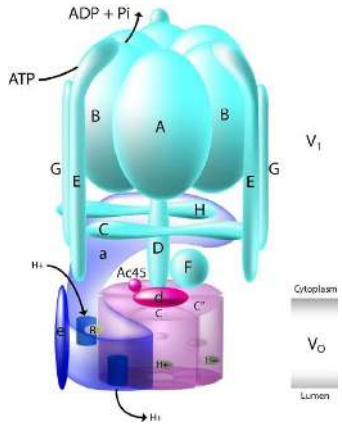


Figure 3.39 - Schematic structure of V-ATPases- Wikipedia

Nerve transmission

Now that you have seen how the Na+/K+ ATPase functions, it is appropriate to discuss how nerve cells use ion gradients created with it to generate and transmit nerve signals. Neurons are cells of the nervous system that use chemical and electrical signals to rapidly transmit information across the body (Figure 3.40). The sensory nerve system links receptors for vision, hearing, touch, taste, and smell to the brain for perception. Motor neurons run from the spinal cord to muscle cells. These neurons have a cell body and a very long, thin extension called an axon, that stretches from the cell body in the spinal cord all the way to the muscles they control. Nerve impulses travel down the axon to stimulate muscle contraction.

Signals travel through neurons, ultimately arriving at junctions with other nerve cells or target cells such as muscle cells. Note that neurons do not make physical contact with each other or with muscle cells. The tiny space between two neurons or between a neuron and a muscle cell is called the synaptic cleft. At the synaptic cleft, the neuron releases neurotransmitters that exit the nerve cell and travel across the junction to a recipient cell where a response is generated. That response may be creating another nerve signal, if the adjacent cell is a nerve cell or it may be a muscular contraction if the recipient is a muscle cell (Figure 3.41).

In considering information movement via nerve cells, then, we will discuss two steps - 1) creation and propagation of a signal in a nerve cell and 2) action of neurotransmitters exiting a nerve cell and transiting a synaptic junction.

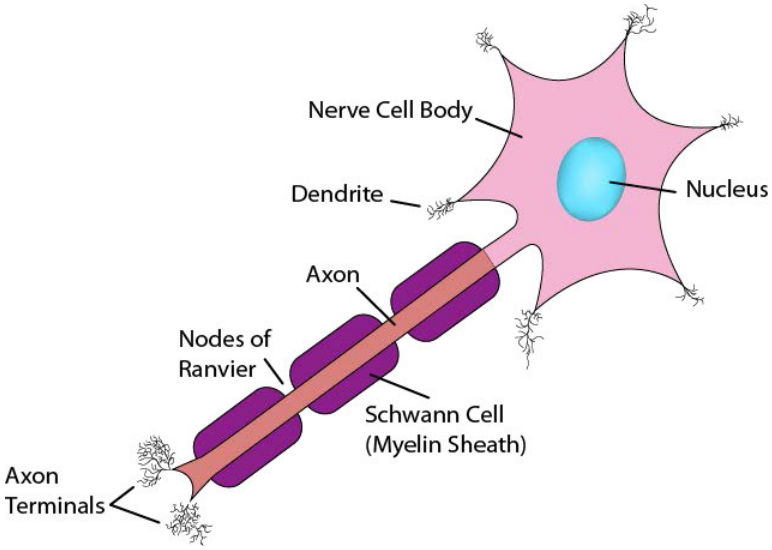


Figure 3.40 - Anatomy of a nerve cell - Image by Pehr Jacobson

Signal source

Creation of a nerve signal begins with a stimulus to the nerve cell. In the case of muscle contraction, the motor cortex of the brain sends signals to the appropriate motor neurons, stimulating them to generate a nerve impulse. How is such an impulse generated?

Resting potential

In the unstimulated state, all cells, including nerve cells, have a small voltage difference (called the resting potential) across the plasma membrane, arising from unequal pumping of ions across the membrane. The Na+/K+ ATPase, for example, pumps sodium ions out of the cell and potassium ions into cells. Since three sodium ions get pumped out for every two potassium ions pumped in, a charge and chemical gradient is created. It is the charge gradient that gives rise to the resting potential.

Altering the gradients of ions across membranes provide the driving force for nerve signals. This happens as a result of opening and closing of gated ion channels. Opening of gates to allow ions to pass through the membrane swiftly changes the ionic balance across the membrane resulting in a new voltage difference called the action potential. It is the action potential that is the impetus of nerve transmission.

Initiation of signal

The signal generated by a motor neuron begins with opening of sodium channels in the membrane of the nerve cell body causing a rapid influx of sodium ions into the nerve cell. This step, called depolarization (Figure 3.42), triggers an electrochemical signal - the action potential. Remember that the Na+/K+ ATPase has created a large sodium gradient, so sodium ions rush into the cell when sodium channels open. After the initial depolarization, potassium channel gates, responding to the depolarization, open, allowing potassium ions to rapidly diffuse out of the cell (remember K+ ions are more abundant inside of the cell). This phase is called the repolarization phase and during it, the sodium gates close.

The rapid exit of potassium ions causes the voltage difference to “overshoot” the resting potential and potassium gates close. This followed by the so-called refractory period, when the Na+/K+ ATPase begins its work to re-establish the original conditions by pumping sodium ions out and potassium ions into the nerve cell. Eventually, the system recovers and the resting potential is re-established. The initiating end of the nerve cell is then ready for another signal.

Propagation of action potential

What we have described here is only the initiation of the nerve signal in one part of the nerve cell. For the signal to be received, the action potential must travel the entirety of the length of the nerve cell (the axon) and cause a chemical signal to be released into the synaptic cleft to get to its target. Propagating the nerve signal (action potential) in the original nerve cell is the function of all of the rest of the gated ion channels (Figure 3.43) positioned on the sides of the nerve cell. The sodium and potassium gates involved in propagation of the signal all act in response to voltage changes created by the electrochemical gradient moving down the nerve cell (Figure 3.44). Remember that opening of the initial gates at initiation of the signal created an influx of sodium ions and an efflux of potassium ions.

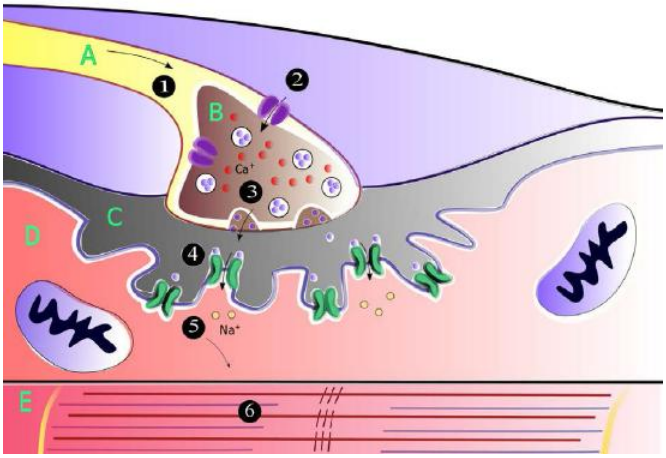


Figure 3.41 - (1) The action potential reaches the axon terminal; (2) voltage-dependent calcium gates open - calcium enters the axon terminal; (3) neurotransmitter vesicles fuse with the presynaptic membrane and release acetylcholine (ACh); (4) ACh binds to postsynaptic receptors on the sarcolemma; (5) ion channels open in response and allow sodium ions to flow into the muscle cell; (6) The flow of sodium ions into the muscle cell generates an action potential which travels to the myofibril and results in muscle contraction. - Wikipedia

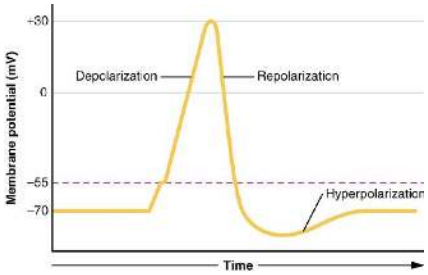


Figure 3.42 - Depolarization and repolarization of a nerve cell - Wikipedia

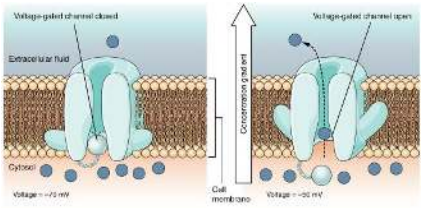


Figure 3.43 - Voltage gated ion channels - Wikipedia

https://chem.libretexts.org/@go/page/354313

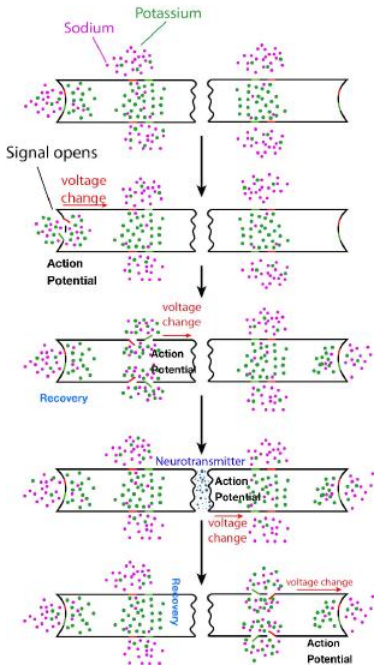


Figure 3.44 - Action potential moving across a synaptic junction of two nerve cells - Image by Pehr Jacobson

Moving signal

This chemical and electrical change that creates the action potential leaves the end of the nerve cell where it started and travels down the axon towards the other end of the nerve cell. Along the way, it encounters more sodium and potassium gated channels. In each case, these respond simply to the voltage change of the action potential and open and close, exactly in the same way the gates opened to start the signal. Thus, a rapid wave of increasing sodium ions and decreasing potassium ions moves along the nerve cell, propagated (and amplified) by gates opening and closing as the ions and charges move down the nerve cell. Eventually, the ionic tidal wave reaches the end of the nerve cell (axon terminal) facing the synaptic cleft.

Crossing the synaptic cleft

For the signal to be received by the intended target (postsynaptic cell) from the originating neuron (presynaptic neuron), it must cross the synaptic cleft and stimulate the neighboring cell (Figure 3.45). Communicating information across a synaptic cleft is the job of neurotransmitters. These are small molecules synthesized in nerve cells that are packaged in membrane vesicles called synaptic vesicles in the nerve cell. Neurotransmitters come in all shapes and chemical forms, from small chemicals like acetylcholine to peptides like neuropeptide Y. The most abundant neurotransmitter is glutamate, which acts at over 90% of the synapses in the human brain.

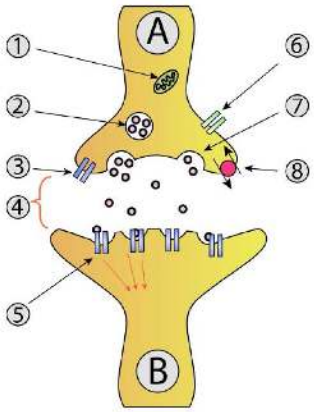


Figure 3.45 - A) Pre-synaptic neuron; B) post-synaptic neuron; 1) Mitochondria; 2) synaptic vesicle with neurotransmitters; 3) autoreceptor; 4) synapse with neurotransmitter released (serotonin); 5) postsynaptic receptors activated by neurotransmitter; 6) calcium channel; 7) exocytosis of a vesicle; 8) recaptured neurotransmitter. Wikipedia

Movie 3.2 - Movement of an action potential down a nerve cell - Wikipedia

Into the cleft

As the action potential in the presynaptic neuron approaches the axon terminus, synaptic vesicles begin to fuse with the membrane and their neurotransmitter contents spill into the synaptic cleft. Once in the cleft, the neurotransmitters diffuse, some of them reaching receptors on the postsynaptic cell. Binding of the neurotransmitter to the receptors on the membrane of the postsynaptic cell stimulates a response.

For motor neurons, the postsynaptic cell will be a muscle cell, and the response will be muscle contraction/relaxation. At this point, the originating nerve cell has done its job and communicated its information to its immediate target. If the postsynaptic cell is a nerve cell, the process repeats in that cell until it gets to its destination.

N

Distance Ed

To the tune of “Mister Ed”

Metabolic Melodies Website HERE

A course is a source,

of course, of course

Of all of the knowledge that we endorse

A major force for better/worse is the campus Distance Ed

It’s true to outsource a college course

There are a few standards to be enforced

The long and short’s we reinforce the campus Distance Ed

Bridge

A classroom class meets every week the same time every day

But Distance Ed is most unique - its flexible schedule’s okay

E-course is a source, of course, of course

Of online assistance for lab reports

You’re not enrolled in an online course?

Then sign up for this!

“You’ll love Distance Ed”

Recording by David Simmons

Lyrics by Kevin Ahern
Recording by David Simmons Lyrics by Kevin Ahern

313

It's one o'clock and
Ahern's talkin'
Henderson and
Hasselbalch and
pKa's and
Buffers I should know
This song's for BB three five oh

I hope that maybe
He'll think the way we
Wrote our answers
Wasn't crazy
I really need the
Partial credit - so
This song's for BB three five oh

It's really groovy
That it improves me
Watching lectures
In Quicktime movies
I really need to
Go and download those
Podcasts for BB three five oh

This Song's For BB 3-5-0
To the tune of "This Land is My Land"

Metabolic Melodies Website HERE

I'm feeling manic
I'm in a panic
I'd better study
My old organic
It has reactions
That I need to know
This song's for BB three five oh

I know he said it
That's why I dread it
'cause I skipped Friday's
Extra credit
'twil pro'bly haunt me
That lowly ze-ro
Grade in BB three five oh

It could be steric
Or esoteric
That carbons get so
Anomeric
I'm too hysteric
Better let it go
This song's for BB three five oh

Recording by Tim Karplus

Lyrics by Kevin Ahern
Recording by Tim Karplus Lyrics by Kevin Ahern

This page titled 5.4.1: Optional deep dive in to membrane transport is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **3.2: Transport in Membranes** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

5.5: Other Considerations in Membranes

Source: BiochemFFA_3_3.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

There are many functions and factors relating to cell membranes that don't fit into broad categories. Those items will be the focus of this section.

Endocytosis

Besides transporter proteins and ion channels, another common way for materials to get into cells is by the process of endocytosis. Endocytosis is an alternate form of active transport for getting materials into cells. Some of these processes, such as phagocytosis, are able to import much larger particles than would be possible via a transporter protein. Like transporter proteins, endocytosis uses energy for the purpose (though it is not as visible as with protein transporters), but unlike protein transporters, the process is not nearly as specific for individual molecules.

As a result, the process usually involves the importation of many different molecules each time it occurs. The list of compounds entering cells in this way includes LDLs and their lipid contents, but it also include things like iron (packaged in transferrin), vitamins, hormones, proteins, and even some viruses sneak in by this means. There are three types of endocytosis we will consider (Figure 3.53).

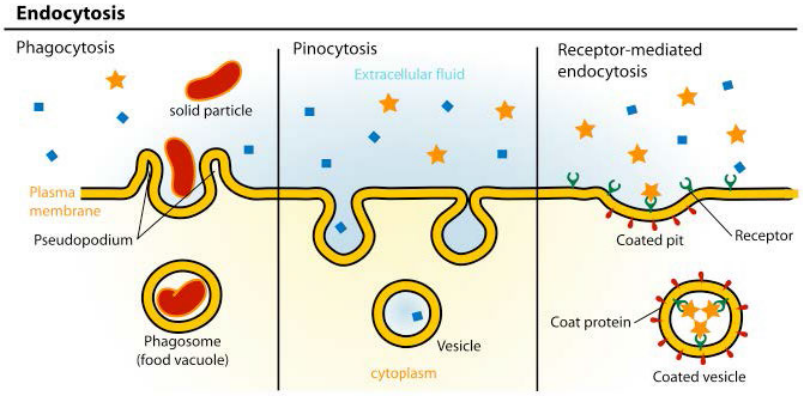


Figure 3.53 - Three types of endocytosis

Receptor mediated endocytosis

The process of receptor mediated endocytosis is a relatively specific means of bringing molecules into cells because it requires the incoming material to be somehow associated with a specific cell surface receptor. In the example of Figure 3.53, the receptor is the cellular LDL receptor. Clathrin-coated invaginations, as shown in the figure are known as "coated pits." The mechanism proceeds with an inward budding of the plasma membrane receptor (coated vesicles). Binding of the ligand (ApoB-100 of the LDL, for example, in Figure 3.54) to the LDL receptor leads to formation of a membrane invagination. The absorbed LDL particle fuses to form an early endosome (Figure 3.55) and contents are subsequently sorted and processed for use by the cell.

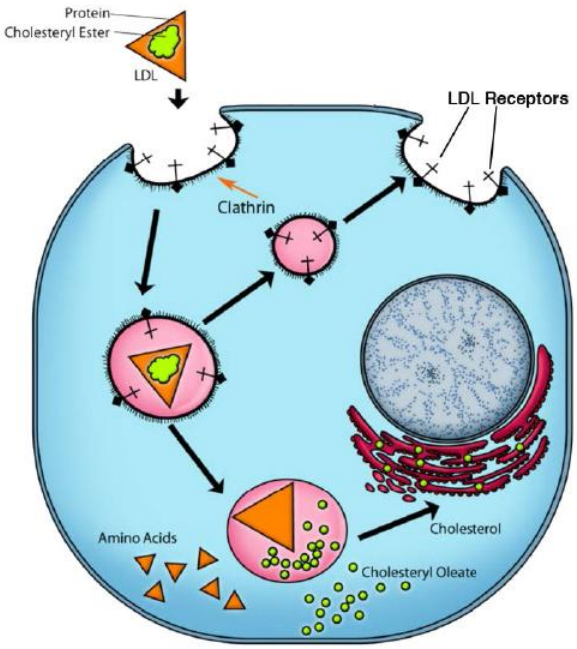


Figure 3.54 - Overview of clathrin-based receptor mediated endocytosis - Image by Aleia Kim

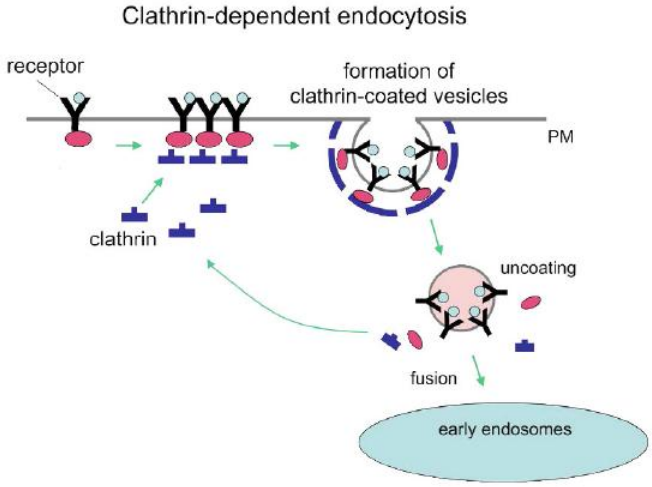


Figure 3.55 - Clathrin-mediated endocytosis of receptors - Wikipedia

The components from the coated vesicle are recycled to the plasma membrane for additional actions. Receptor mediated endocytosis can also play a role in internalization of cellular receptors that function in the process of signaling. Here, a receptor bound to a ligand is brought into the cell and may ultimately generate a response in the nucleus.

While receptor mediated endocytosis of receptors can have the effect of communicating a signal inwards to the cell, it can also reduce the total amount of signaling occurring, since the number of receptors on the cell surface is decreased by the process.

Non-clathrin endocytosis

There are three types of endocytosis occurring in cells that do not involve clathrin. They are 1) caveolae-based endocytosis, 2) macropinocytosis, and 3) phagocytosis. Caveolae-based endocytosis is based on a receptor molecule known as caveolin. Caveolins are a class of integral membrane proteins that compartmentalize and concentrate signaling molecules in the process of endocytosis. They are named for the cave-like caveolae structures of the plasma membrane where they are found.

Caveolins

Caveolins have affinity for cholesterol and associate with it in the membrane of cells, causing the formation of membrane invaginations of about 50 nm. The caveolin proteins can oligomerize and this is important for the coating and formation of the cave-like structures.

There are three caveolin genes found in vertebrate cells, CAV1, CAV2, and CAV3. Down-regulation of caveolin-1 results in less efficient cellular migration in vitro. Caveolins are implicated in both formation and suppression of tumors. High expression of them inhibits cancer-related growth factor signaling pathways, but some caveolin-expressing cancer cells are more aggressive and metastatic, possible due to an enhanced capacity for anchorage-independent growth.

Macropinocytosis

A phenomenon known as “cell drinking,” macropinocytosis literally involves a cell “taking a gulp” of the extracellular fluid. It does this, as shown in Figure 3.56, by a simple invagination of ruffled surface features of the plasma membrane. A pocket results, which pinches off internally to create a vesicle containing extracellular fluid and dissolved molecules. Within the cytosol, this internalized vesicle will fuse with endosomes and lysosomes. The process is non-specific for materials internalized.

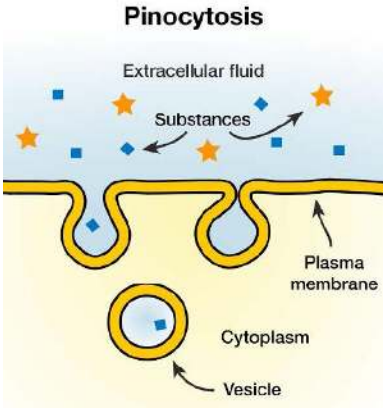


Figure 3.56 - Macropinocytosis

Phagocytosis

Phagocytosis is a process whereby relatively large particles (0.75 µm in diameter) are intenalized. Cells of the immune system, such as neutrophils, macrophages, and others, use phagocytosis to internalize cell debris, apoptotic cells, and microorganisms.

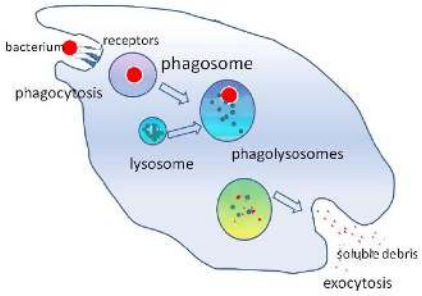


Figure 3.57 - Generalized scheme for phagocytosis of a bacterium - Wikipedia

The process operates through specific receptors on the surface of the cell and phagocytosing cell engulfs its target by growing around it. The internalized structure is known as a phagosome, which quickly merges with a lysosome to create a phagolysosome (Figure 3.58), which subjects the engulfed particle to toxic conditions to kill it, if it is a cell, and/or to digest it into smaller pieces. In some cases, as shown in the figure, soluble debris may be released by the phagocytosing cell.

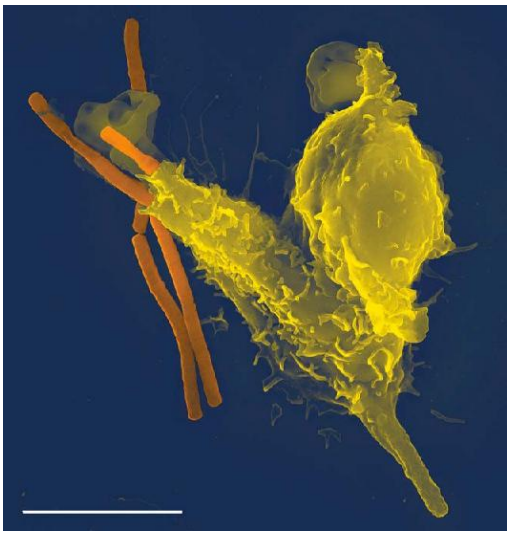


Figure 3.58 - Phagocytosis by a neutrophil (yellow) of an Anthrax bacillus (orange) - Wikipedia

Endosomes

Internalized material from endocytosis that doesn't involve phagocytosis passes through an internalized structure called an endosome. Endosomes are membrane bounded structures inside of eukaryotic cells that play a role in endocytosis (Figure 3.59). They have a sorting function for material internalized into the cell, providing for retrieval of materials not destined for destruction in the lysosomes. LDLs, for example, are targeted after endocytosis to the endosomes for processing before part of them is delivered to the lysosome. The endosomes can also receive molecules from the trans-Golgi network. These can be delivered to the lysosomes, as well, or redirected back to the Golgi. Endosomes come in three forms - 1) early, 2) late, and 3) recycling.

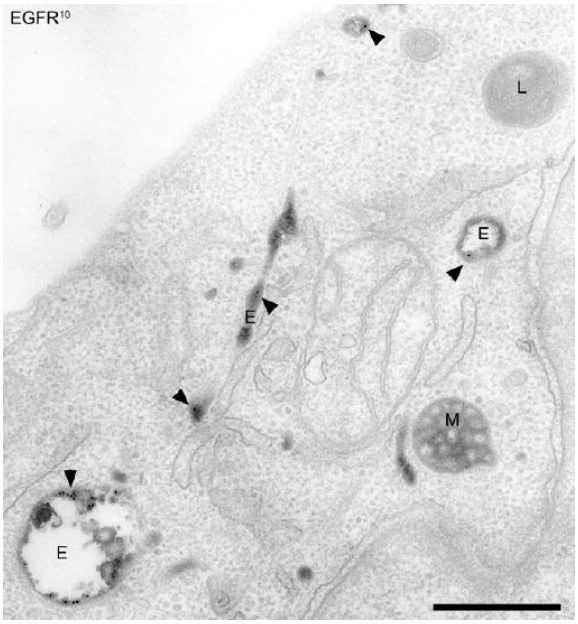


Figure 3.59 - Internalization of the epidermal growth factor receptor (EGFR) into endosomes. Early (E) and late (M) endosomes and lysosomes (L) are labeled. - Wikipedia

Exocytosis

The process of exocytosis is used by cells to export molecules out of cells that would not otherwise pass easily through the plasma membrane. In the process, secretory vesicles fuse with the plasma membrane and release their contents extracellularly. Materials, such as proteins and lipids embedded in the membranes of the vesicles become a part of the plasma membrane when fusion between it and the vesicles occurs.

Membrane fusion

Fusion is a membrane process where two distinct lipid bilayers merge their hydrophobic cores, producing one interconnected structure. Membrane fusion involving vesicles is the mechanism by which the processes of endocytosis and exocytosis occur.

When the fusion proceeds through both leaflets of both bilayers, an aqueous bridge results and the contents of the two structures mix. Common processes involving membrane fusion (Figure 3.60) include fertilization of an egg by a sperm, separation of membranes in cell division, transport of waste products, and neurotransmitter release (Figure 3.61). Artificial membranes such as liposomes can also fuse with cellular membranes. Fusion is also important for transporting lipids from the point of synthesis inside the cell to the membrane where they are used. Entry of pathogens can also be governed by fusion, as many bilayer-coated viruses use fusion proteins in entering host cells.

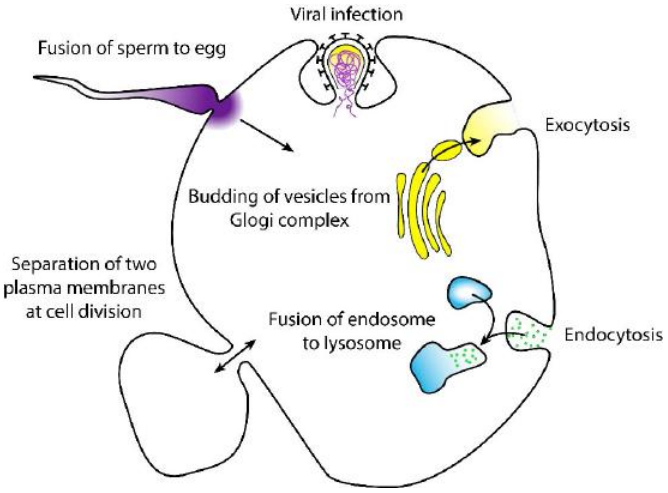


Figure 3.60 - Cell membrane fusions - Image by Pehr Jacobson

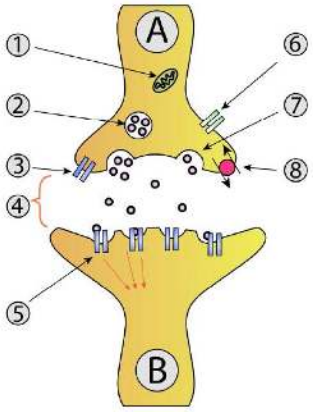


Figure 3.61 - Release of neurotransmitters (small circles) from presynaptic neuron A to postsynaptic neuron B. 1 = Mitochondrion / 2 = Synaptic vesicle with neurotransmitter / 3 = Autoreceptor / 4 = Synaptic cleft / 5 = Neurotransmitter receptor / 6 = Calcium channel / 7 = Fused vesicle releasing neurotransmitter / 8 = Neurotransmitter re-uptake pump - Wikipedia

SNARE proteins

Mediation of fusion of vesicles in exocytosis is carried out by proteins known as SNAREs (Soluble NSF Attachment Protein REceptor). This large superfamily of proteins spans a wide biological range, from yeast to mammals. Common vesicle fusions occur when synaptic vesicles dock with neurons (Figure 3.61) and release neurotransmitters. These are well-studied. The SNAREs involved in this process can be proteolytically cleaved by bacterial neurotoxins that give rise to the conditions of botulism and tetanus.

SNAREs are found in two locations. v-SNAREs are found in the membranes of transport vesicles during the budding process, whereas t-SNARES can be found in the membranes of targeted compartments.

The act of vesicle fusion coincides with increases of intracellular calcium. Fusion of synaptic vesicles in neurotransmission results in activation of voltage-dependent calcium channels in the targeted cell. Influx of calcium helps to stimulate vesicle fusion. In the endocrine system, binding of hormones to G protein coupled receptors activate the IP3/DAG system to increase levels of calcium.

In the process of membrane fusion (Figure 3.62), the v-SNAREs of a secretory vesicle (upper left) interact with the t-SNAREs of a target membrane (bottom). The v- and t-SNAREs "zipper" themselves together to bring the membrane vesicle and the target membrane closer together.

Zippering also causes flattening and lateral tension of the curved membrane surfaces, favoring hemifusion of the outer layers of each membrane. Continued tension results in subsequent fusion of the inner membranes as well, yielding opening of the contents of the vesicle chamber to its target (usually outside the cell).

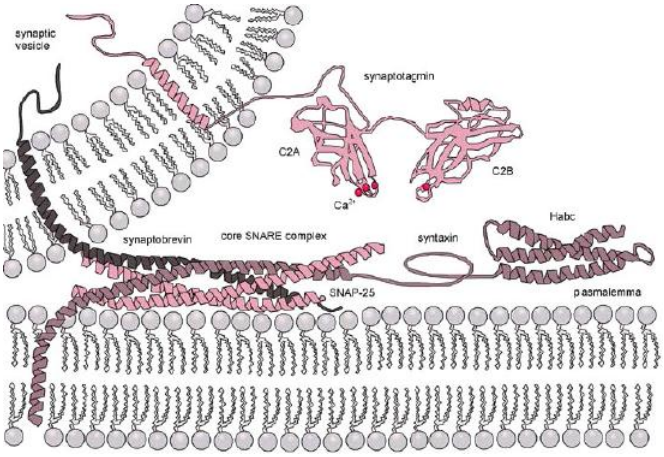


Figure 3.62 - Proteins involved in vesicle fusion in neurotransmission. A SNARE complex between α-helices of synaptobrevin, syntaxin and SNAP-25 intertwine and "zip" membranes together. Synaptotagmin is a calcium sensor regulating the process of zipping - Wikipedia

Shuttles

Another way to transport items across a membrane for which there is no specific transport system available is the use of shuttles. Shuttles are important when there is no transport mechanism for moving material across a membrane for which no transport system exists.

A great example is NADH. NADH is an important electron carrier that is produced in the cytoplasm and mitochondria of the cell. NADH produced in the mitochondrion goes directly to the electron transport system and delivers electrons to Complex I. NADH produced in the cytoplasm (such as from glycolysis) does not have this option, since the inner membrane of the mitochondrion is impermeable to the molecule and no transporter exists to move it across. The important part of the NADH is its electron cargo, so cells have evolved two ways to move the electrons into the mitochondrial matrix apart from NADH.

Both methods involve shuttles. In each case, an acceptor molecule receives electrons from NADH and the reduced form of the acceptor molecule is transported. It gets transported into the matrix where it is oxidized (electrons are lost) and then donated to the electron transport system.

Glycerol phosphate shuttle

The first of these methods is the least efficient, but it is rapid. It found commonly in muscles which have needs for rapid energy and brain tissue. This shuttle is referred to as the glycerol phosphate shuttle and is shown in Figure 3.63. It operates in the intermembrane space between the inner and outer mitochondrial membranes. The outer mitochondrial membrane is very porous, allowing many materials to pass freely through it. In the intermembrane space, the cytoplasmic enzyme, glyceraldehyde-3-phosphate dehydrogenase (cGPD) catalyzes transfer of electrons from NADH to dihdydroxyacetone phosphate (#2 in the figure), yielding NAD+ and glyceraldehyde-3-phosphate (#1 in the figure). The glyceraldehyde-3-phosphate then binds to a glyceraldehyde-3-phosphate dehydrogenase (mGPD) embedded in the outer portion of the inner mitochondrial membrane. mGPD catalyzes the transfer of electrons from glyceraldehyde-3-phosphate to FAD, producing dihycroxyacetone phosphate and FADH2. FADH2 then transfers its electrons to the electron transport system through CoQ (Q above), forming CoQH2 (QH2 above). As will

be discussed in the section on electron transport, this is not an efficient shuttle system because it does not result in production of as much ATP as occurs when electrons are transferred to NAD+ instead of FAD.

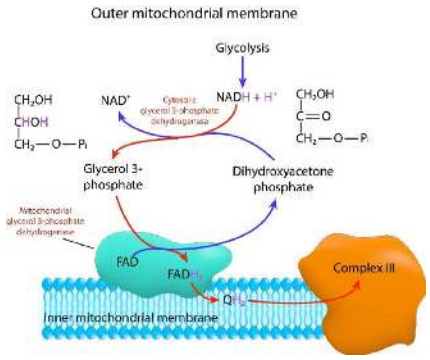


Figure 3.63 - Glycerol phosphate shuttle system in the intermembrane space of a mitochondrion - Image by Pehr Jacobson

Malate-aspartate shuttle

A more efficient system of transferring electrons is the malate-aspartate shuttle and it is shown in Figure 3.64. As is apparent in the figure, this shuttle involves more steps than the glycerol phosphate shuttle, but the advantage of the malate-aspartate shuttle is that it is more efficient. NADH outside of the mitochondrion transfers its electrons to the shuttle and then NADH is re-made on the inside of the shuttle. No energy is expended in the process.

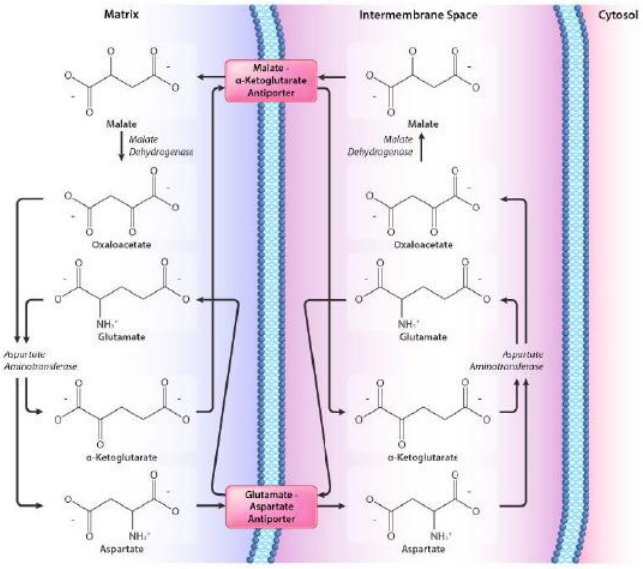


Figure 3.64 - The malate aspartate shuttle - Image by Aleia Kim

When NADH accumulates in the cytoplasm, it moves to the intermembrane space where the enzyme malate dehydrogenase catalyzes the transfer of electrons to oxaloacetate to yield NAD+ and malate. A transport system for malate moves malate into the mitochondrial matrix in exchange for α-ketoglutarate.

Inside the mitochondrion, malate is reoxidized to oxaloacetate and electrons are given to NAD+ to recreate NADH. NADH then donates electrons to Complex I of the electron transport system. That's really all there is to the shuttle. The remaining steps are simply to balance the equation of the process. Oxaloacetate accepts an amine group from glutamic acid to yield aspartic acid and α-ketoglutarate. Aspartate then moves out of the mitochondrion through an antiport transport protein that swaps it for glutamate. A series of reactions in the intermembrane space balance the equation.

It is easy to get lost in the mess of balancing equations. The most important thing to understand here is that oxaloacetate accepts electrons on the outside to become malate which is the carrier of electrons across the membrane. Once inside the matrix, malate is converted back to oxaloacetate and its electrons are given to NAD+, forming NADH. Everything else is simple equation balancing.

Acetyl-CoA shuttle

Another kind of shuttle also involves the mitochondrion and in this case, the item being moved is a molecule, not a pair of electrons. The molecule of interest here is acetyl-CoA, for which no transport system operates, but which is needed in the cytoplasm for fatty acid synthesis when the cell has abundant energy.

Acetyl-CoA is mostly in the mitochondrion and so long as the citric acid cycle is operating efficiently, its concentration is relatively stable. However, when the citric acid cycle slows, acetyl-CoA and the citrate made from it in the cycle begin to accumulate.

A membrane transport system for citrate exists, so it gets moved out to the cytoplasm. In the cytoplasm, an enzyme known as citrate lyase cleaves citrate to acetyl-CoA and oxaloacetate. Oxaloacetate can be reduced to malate and moved back into the mitochondrion.

As for acetyl-CoA, the more of that cells have in the cytoplasm, the more likely they will begin making fatty acids and fat, since acetyl-CoA is the starting material for fatty acid synthesis, which occurs in the cytoplasm. When does this process occur? As noted above, it occurs when the citric acid cycle stops and this occurs when levels of NADH and FADH2 increase. These, of course, increase when one is not burning off as many calories as one is consuming as a byproduct of respiratory control. Lack of exercise leads to higher levels of reduced electron carriers.

Cell junctions

Cells in multicellular organisms are in close contact with each other and links between them are called junctions. In vertebrate organisms, there are three main types of cell junctions and one of them (gap junctions) is important for movement of materials between cells. The three types are

1. Gap junctions
2. Adherens junctions, (Anchoring Junctions, desmosomes and hemidesmosomes)
3. Tight junctions

Cell junctions in multicellular plants are structured differently from those in vertebrates and are called plasmodesmata. They too function in exchange of materials between individual cells.

Gap junctions

Gap junctions are specialized structures made up of two sets of structures called connexons (one from each cell - see Figure 3.65) directly link the cytoplasms of the connected cells. Gap junctions are regulated to control the flow of molecules, ions, and electrical impulses between cells. In plants, similar structures known as plasmodesmata traverse the cell wall (Figure 3.66) and perform similar functions.

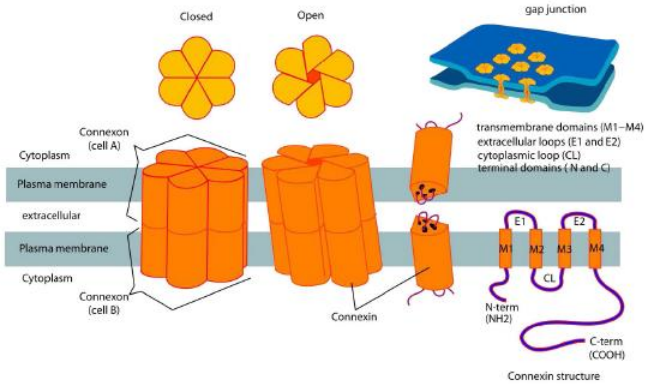


Figure 3.65 - Structure of connexons joining two cells. Bundles of six copies of connexin proteins in the plasma membrane of each cell comprise the connexon structures

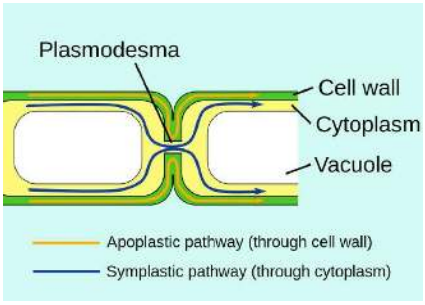


Figure 3.66 - Two means of intercellular communication in plant cells - apoplastic pathway (through cell wall) and symplastic pathway (through the plasodesma)

Adherens junctions

Adherens junctions (Figure 3.67) are protein complexes on the cytoplasmic side of the cell membranes of epithelial and endothelial tissues that link cells to each other or to the extracellular matrix. They correspond to the fascia adherens found in non-epithelial/non-endothelial cells.

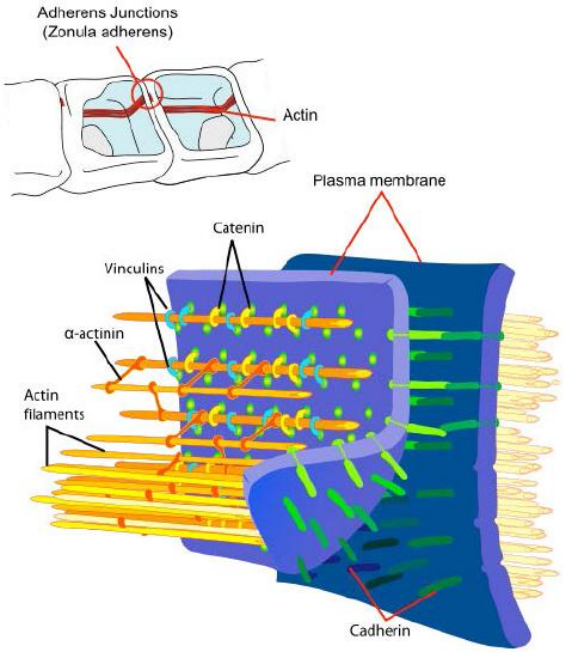


Figure 3.67 - Adherens junction

Adherens junctions contain the following proteins - 1) α-catenin (binds cadherin through β-catenin); 2) β-catenin (attachment for α-catenin to cadherin; 3) γ-catenin (binds to cadherin); 4) cadherins (group of transmembrane proteins that dimerize with cadherins on adjacent cells; 5) p120 (also called Δ-catenin - binds to cadherin); 6) plakoglobin (catenin family protein homologous to and acting like β-catenin); 7) actin; 8) actinin; and 9) vinculin. Adherens junctions may help to maintain the actin contractile ring which forms in the process of cytokinesis.

Tight junctions

Tight junctions (Figure 3.68) are a network of protein strands that seal cells together and restrict the flow of ions in the spaces between them. The effect of their structure is to restrict the movement of materials through tissues by requiring them to pass through cells instead of around them. Tight junctions join together the cytoskeletons of cells and through their structure maintain their apical and basolateral polarity.

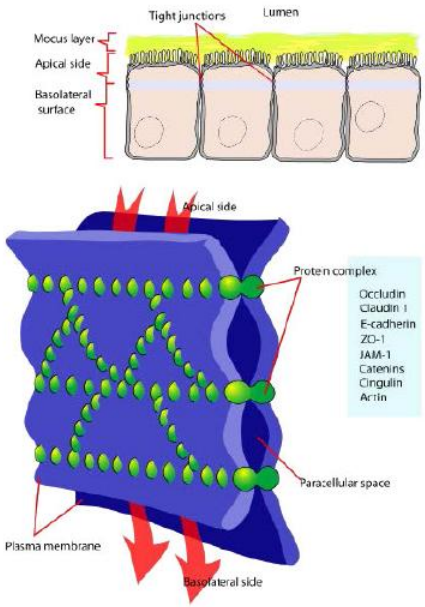


Figure 3.68 - Tight junctions

GPI anchors

Membrane proteins attached to glycosylphosphatidylinositol (also known as a GPI anchor) are referred to as being glypiated. The proteins, which play important roles in many biochemical processes, are attached to the GPI anchor at their carboxyl terminus. Phospholipases, such as phospholipase C can cut the bond between the protein and the GPI, freeing the protein from the outer cell membrane. Proteins destined to be glypiated have two signal sequences. They are 1) An N-terminal signal sequence and 2) A C-terminal signal sequence that is recognized by a GPI transamidase (GPIT). The N-terminal signal sequences is responsible for directing co-translational transport into the endoplasmic reticulum. The C-terminal sequence is recognized by GPI transamidase, which links the carboxy terminus of a protein to the GPI anchor.

Liposomes

The spontaneous ability of phosphoglycerolipid and sphingolipid compounds to form lipid bilayers is exploited in the formation of artificial membranous structures called liposomes (Figure 3.69). Liposomes are useful for delivering their contents into cells via membrane fusion. In the figure, items targeted for delivery to cells would be encased in the middle circular region of the liposome and when the liposome fuses with the cell membrane, it will deliver the contents directly into the cytoplasm.



Figure 3.70 - Formation of liposomes from phospholipids in water - Image by Pehr Jacobson

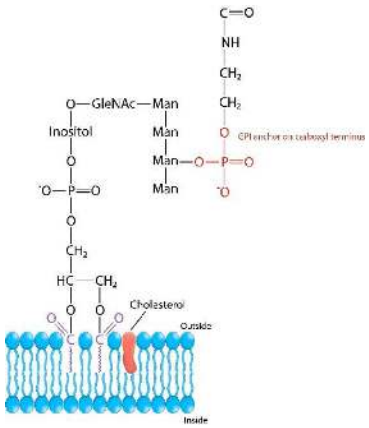


Figure 3.69 - A glypiated protein linked to a membrane-embedded inositol-based molecule. The protein portion is above the red phosphate ion - Image by Pehr Jacobson

Hydropathy index

The interior portion of the lipid bilayer is very hydrophobic, which makes it very restrictive to movement of ions and polar substances across it. This property also places limitations on the types of amino acids that will interact with it as well. Because of this, transmembrane protein domains found in integral membrane proteins are biased in the amino acids that interact with either the lipid bilayer or the aqueous material on either side of it.

Amino Acid	Hydropathy Index
Alanine	1.8
Arginine	−4.5
Asparagine	−3.5
Aspartic acid	−3.5
Cysteine	2.5
Glutamic acid	−3.5
Glutamine	−3.5
Glycine	−0.4
Histidine	−3.2
Isoleucine	4.5
Leucine	3.8
Lysine	−3.9
Methionine	1.9
Phenylalanine	2.8
Proline	−1.6
Serine	−0.8
Threonine	−0.7
Tryptophan	−0.9
Tyrosine	−1.3
Valine	4.2

Figure 3.71 - Hydropathy index for amino acids. More positive values indicate higher hydrophobicity. Wikipedia

Hydrophobic amino acids are found within the bilayer, whereas hydrophilic amino acids are found predominantly on the surfaces. An additional clue to identifying membrane crossing regions of a protein is that tryptophan or tyrosine is commonly positioned at non-polar/polar interface(s) of the lipid bilayer for integral proteins. Such an organization of amino acids can be recognized by computer analysis of amino acid sequences using what is called a hydropathy index/score (Figure 3.71). Though the names and the scorings vary, the idea is to assign a number (usually positive) to amino acid side chains with higher hydrophobicity and negative to those that are ionic. With these scores, a computer program can easily find the average scores of short amino acid segments (say 3 amino acids long) and then plot those values on a graph of hydrophobicity index versus position in polypeptide chain. Doing that for a transmembrane protein such as glycophorin results in the plot shown in Figure 3.72. It is apparent in the analysis that this is a transmembrane protein that has seven domains crossing the lipid bilayer, as labeled.

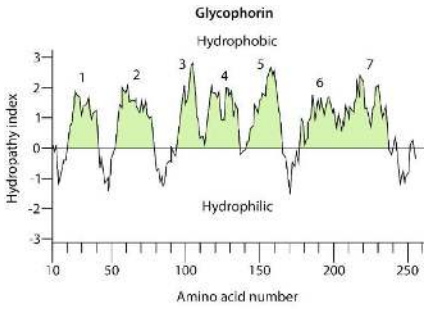


Figure 3.72 - Hydropathy index plot for glycophorin. Each lipid bilayer-crossing domain noted with a number - Image by Pehr Jacobson

Cell walls

Cells walls are found in many cells, including plants, fungi, and bacteria, but are not found in animal cells. They are designed to provide strength and integrity and at least some protection against bursting from osmotic pressure (Figures 3.73-3.75).

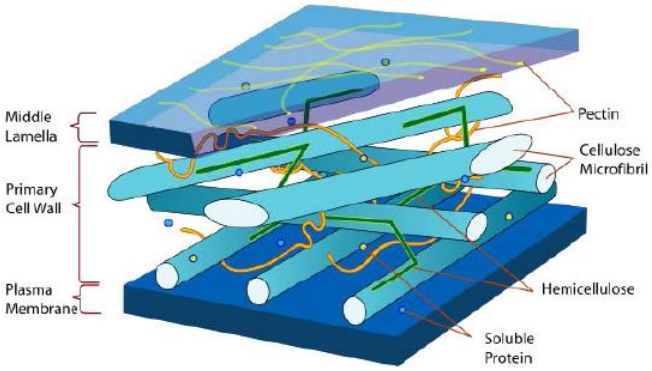


Figure 3.73 - Plant cell wall. Direction of the cytoplasm is down

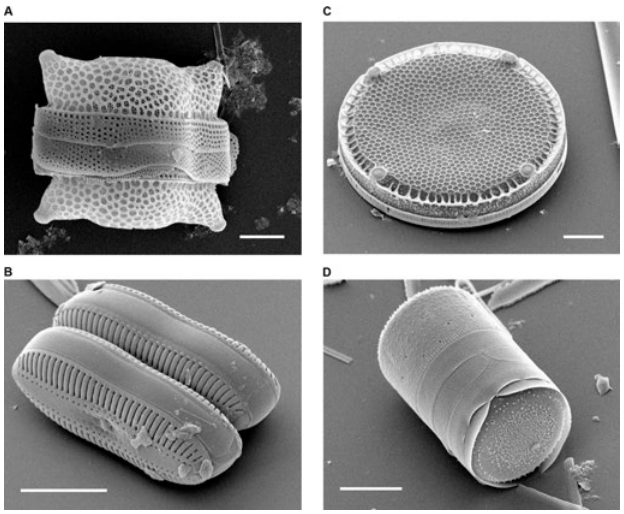


Figure 3.74 - Cell walls of diatoms - Wikipedia

Gram positive bacteria (Figure 3.75) have the simplest cell wall design. Moving from outside the cell towards the cytoplasm there is an outer peptidoglycan layer for the cell wall followed by a periplasmic space, a plasma membrane, and then the cytoplasm. Gram negative bacteria add a second protective layer external to all of this, so for them, starting at the outside and moving inwards, one encounters an outer lipopolysaccharide layer, a periplasmic space, the peptidoglycan cell wall, a second periplasmic space, a plasma membrane and then the cytoplasm.

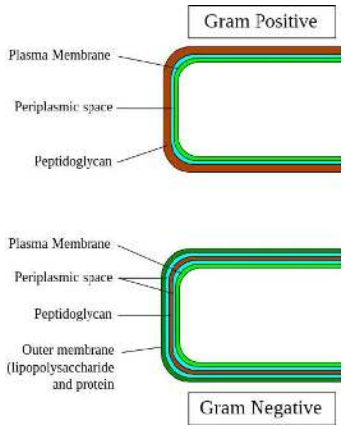


Figure 3.75 - Gram positive versus Gram negative bacteria cell coverings - Wikipedia

Herbaceous plants have a rigid outer cell wall (primarily composed of cellulose, hemicellulose, and pectin) and an inner plasma membrane. Woody plants add a second level of wall with lignin between the cellulosic wall and the plasma membrane of herbaceous plants.

BB Wonderland

To the tune of “Winter Wonderland”

Metabolic Melodies Website HERE

Milam Hall - It’s 12:30

And Ahern’s gettin’ wordy

He walks to and fro’

While not talkin’ slow

Givin’ it to B-B-4-5-0

I was happy when the term got started

Lecture notes and videos galore

MP3s got added to my iPod

But recitations sometimes were a bore

And exams bit me roughly

When the curve turned out ugly

I don’t think it’s so

My scores are too low

Slidin’ by in B-B-4-5-0

Final-LY there’s an examination

On December 9th at 6:00 pm

I’ll have my card packed with information

So I don’t have to memorize it then

And I’ll feel like a smarty

With my jam-packed note-cardy

Just one more to go

And then ho-ho-ho

I'll be done with B-B-4-5-0

Recording by David Simmons

Lyrics by Kevin Ahern
Recording by David Simmons Lyrics by Kevin Ahern

334

Thank God There's a Video

To the tune of "Thank God I'm a Country Boy"

Metabolic Melodies Website HERE

There's a bundle of things a student oughta know

And Ahern's talk isn't really very slow

Learnin' ain't easy / the lectures kinda blow

Thank God there's a video

Well we've gone through the cycles and their enzymes too
Studying the regulation everything is new
I gotta admit that I haven't got a clue
What am I gonna do?

So I got me a note card and bought me a Stryer
Got the enzymes down and the names he requires
I hope that I can muster up a little more desire
Thank God there's a video

Just got up to speed about the N-A-D
Protons moving through Complex Vee
Electrons dance in the cytochrome C
Gotta hear the MP3

Fatty acid oxidation makes acetyl-CoA
Inside the inner matrix of the mitochondri-ay
It's very complicated, I guess I gotta say
Thank God there's a video

So I got me a note card and bought me a Stryer
Got the enzymes down and the names he requires
I hope that I can muster up a little more desire
Thank God there's a video

Replication's kind of easy in a simple kind of way
Copyin' the bases in the plasmid DNAs
Gs goes with Cs and Ts go with As
Thanks to polymerase

And the DNA's a template for the RNA
Helices unwinding at T-A-T-A
Termination happens, then the enzyme goes away
Don't forget the poly-A

So I got me a note card and bought me a Stryer
Got the enzymes down and the names he requires

I think that I can muster up a little more desire
Thank God there's a video

Recording by David Simmons

Lyrics by Kevin Ahern
Recording by David Simmons Lyrics by Kevin Ahern

This page titled 5.5: Other Considerations in Membranes is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **3.3: Other Considerations in Membranes** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

CHAPTER OVERVIEW

6: Energy and Metabolism

6.1: Basics of Energy
6.2: Metabolism - Sugars
6.3: Citric Acid Cycle and Related Pathways
6.4: Electron Transport and Oxidative Phosphorylation
6.5: Fats and Fatty Acids
6.6: Amino Acids and the Urea Cycle
6.7: Other Lipids
6.8: Energy - Photophosphorylation

6: Energy and Metabolism is shared under a not declared license and was authored, remixed, and/or curated by LibreTexts.

6.1: Basics of Energy

Source: BiochemFFA_5_1.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy



Living organisms are made up of cells, and cells contain a large and diverse set of biochemical components. Living cells, though, are not random collections of these molecules. They are extraordinarily organized or "ordered". By contrast, in the nonliving world, there is a universal tendency to increase disorder. Maintaining and creating order in cells takes the input of energy. Without energy input, life is not possible.

Redox Reactions in Biochemistry

Recall from general chemistry that there is potential energy in chemical bonds. Breaking a bond requires energy, making a bond releases energy. When we talk about energy change in a chemical reaction, we compare energy required to break all bonds and energy released from all bonds that are formed. When a chemical reaction involves a transfer of electrons from one atom to another, we call this reaction a red-ox reaction (recall that oxidation involves loss of electrons, and reduction involves the gain of electrons). There is no oxidation without reduction.

The primary mechanism used by non-photosynthetic organisms to obtain energy is to carry out red-ox reactions. In these reactions, carbon is typically oxidized and in aerobic organisms, oxygen is ultimately reduced to form water. The energy released during the oxidation of carbon is “captured” in ATP and can be used later in coupled reactions. The more reduced a carbon atom is, the more energy can be "harvested" from its oxidation. Fatty acids are highly reduced, whereas carbohydrates are moderately so. Complete oxidation of either fatty acid or carbohydrate produces carbon dioxide.

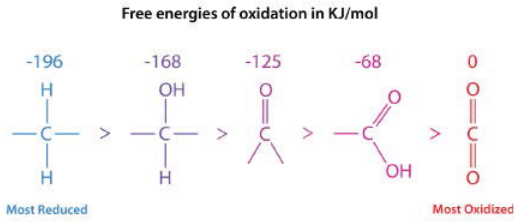


Figure 6.1.1: Five oxidation states of carbon. Image by Pehr Jacobson

In the series shown in Figure 6.1.1, the most reduced form of carbon is on the left. The energy of oxidation of each form is shown above it. The reduction states of fatty acids and carbohydrates can be seen by their formulas.

- Palmitic acid: $C_{16}H_{34}O_2$
- Glucose: $C_6H_{12}O_6$

Palmitic acid only contains two oxygens per sixteen carbons, whereas glucose has six oxygen atoms per six carbons. Consequently, when palmitic acid is fully oxidized, it generates more ATP per carbon (128/16) than glucose (38/6). Fat (specifically, triacyl glycerol) is the primary energy storage material for many organisms because oxidation of fat yields the most ATP per carbon.

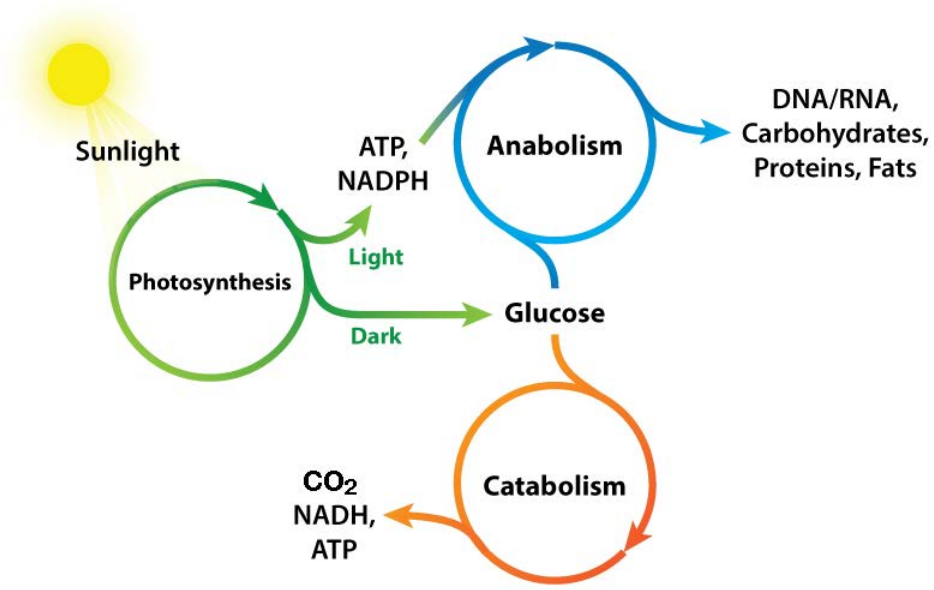


***Figure* 6.1.2**: *Photosynthesis: The primary source of biological energy. Image by Aleia Kim*

Catabolism vs. Anabolism

Biochemical processes that break things down from larger to smaller are called *catabolic processes*. Catabolic processes are often oxidative in nature and energy releasing. Some, but not all, of that energy is captured as ATP. If not all of the energy is captured as ATP, what happens to the rest of it? The answer is simple. It is released as heat and it is for this reason we get hot when we exercise.

By contrast, synthesizing large molecules from smaller ones (for example, making proteins from amino acids) is referred to as anabolism. Anabolic processes are often reductive in nature (Figures 5.3 & 5.4) and require energy input. By themselves, they would not occur, as they are reversing oxidation and decreasing entropy (making many small things into a larger one). To overcome this energy barrier, cells must expend energy. For example, if one wishes to reduce CO_2 to carbohydrate, energy must be used to do so. Plants do this during the dark reactions of photosynthesis (Figure **6.1.3**). The energy source for the reduction is ultimately the sun. The electrons for the reduction come from water, and the CO_2 is removed from the atmosphere and gets incorporated into a sugar.

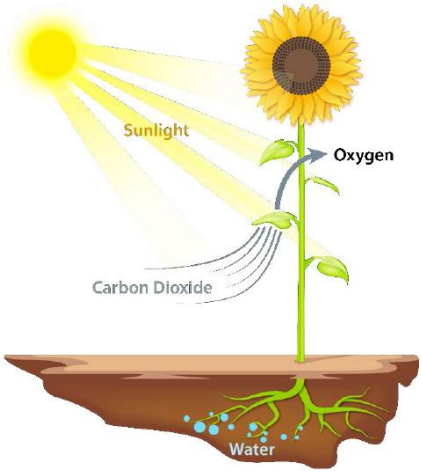


Figure **6.1.3**: Movement of biological energy. Image by Aleia Kim

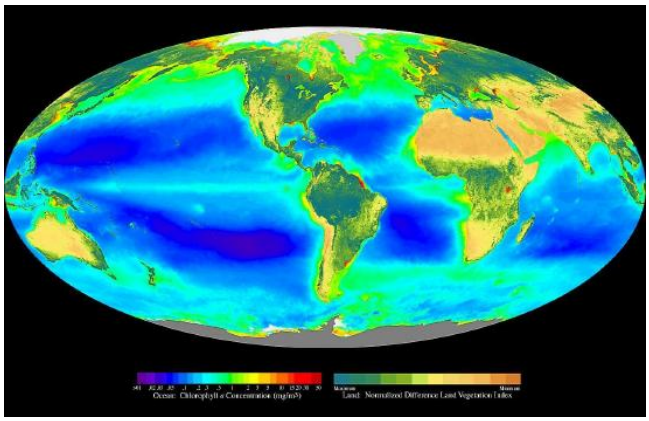


Figure **6.1.4**: Photosynthesis as measured by chlorophyll concentration

Reaction Coupling

The synthesis of the many molecules needed by cells needs the input of energy to occur. Cells overcome this energy obstacle by using ATP to "drive" the reaction (Figure **6.1.6**). Recall that enzymes, biological catalysts, can speed up reactions by lowering the activation energy but do not change the direction of reaction (do not change the overall ΔG). Enzymes can also provide a new path for the reaction by providing a location for additional reactants to bind and undergo chemical transformation. The energy released by an energetically favorable reaction can be used to drive forward an energetically unfavorable reaction. This involves a process called 'coupling'. Coupled reactions rely on linking an energetically favorable reaction (i.e., one with a negative ΔG°') with the reaction requiring an energy input, which has a positive ΔG°'. As long as the overall ΔG°' of the two reactions together is negative, the overall reaction favors the formation of products. Hydrolysis of ATP is a very energetically favorable reaction that is commonly linked to many energy-requiring reactions in cells. Without the hydrolysis of ATP (or GTP, in some cases), many processes would not be feasible in the cell.

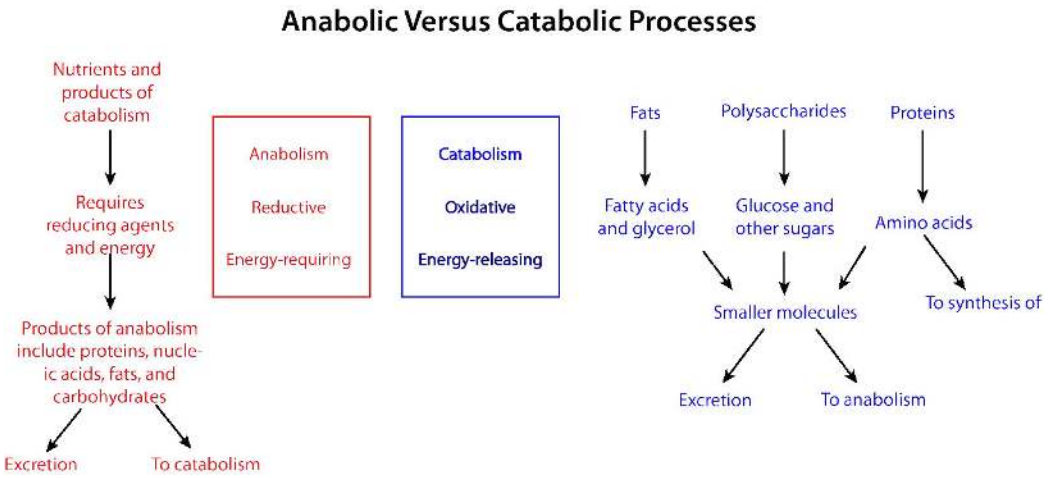


Figure **6.1.5**: Synthesis and breakdown pathways in metabolism. Image by Pehr Jacobson

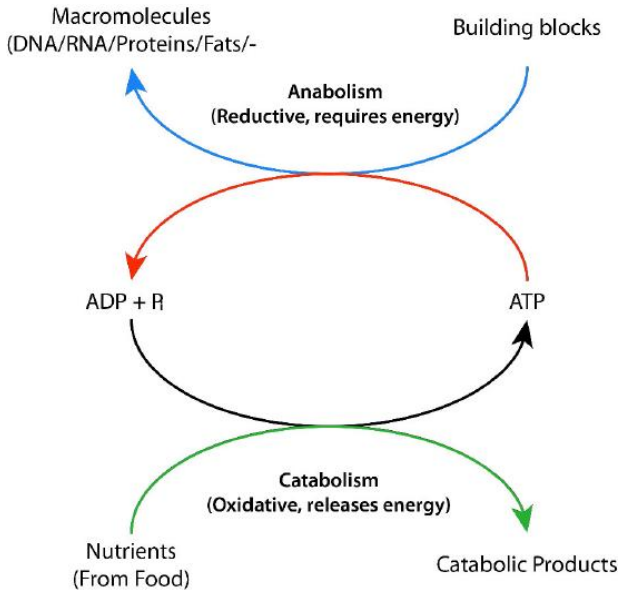


Figure **6.1.6**: Cycling of biological energy via ADP and ATP. Image by Pehr Jacobson

Entropy and energy

Most students who have had some chemistry know about the Second Law of Thermodynamics with respect to the increasing disorder of a system. Cells are very organized or ordered structures, leading some to mistakenly conclude that life somehow violates the second law. In fact, that notion is incorrect. The second law doesn't say that entropy always increases, just that, left alone, it tends to do so, in an isolated system. Cells are not isolated systems, though, in that they obtain energy, either from the sun, if they are autotrophic, or food, if they are heterotrophic.

To counter the universal tendency towards disorder on a local scale requires energy. As an example, take a fresh deck of cards which is neatly aligned with Ace-King-Queen . . . . 4,3,2 for each suit. Throw the deck into the air, letting the cards scatter. When you pick them up, they will be more disordered than when they started. However, if you spend a few minutes (and expend a bit of energy), you can reorganize the same deck back to its previous, organized state. If entropy always increased everywhere, you could not do this. However, with the input of energy, you overcame the disorder. This illustrates an important concept: the cost of fighting disorder is energy.

Biological energy

There are, of course, other reasons that organisms need energy. Muscular contraction, synthesis of molecules, neurotransmission, signaling, thermoregulation, and subcellular movements are examples. Where does this energy come from? The currencies of energy are generally high-energy phosphate-containing molecules. ATP is the best known and most abundant, but GTP is also an

important energy source (an energy source for protein synthesis). CTP is involved in the synthesis of glycerophospholipids and UTP is used for the synthesis of glycogen and other sugar compounds. In each of these cases, the energy is in the form of potential chemical energy stored in the multi-phosphate bonds. Hydrolyzing those bonds releases the energy in them.

Of the triphosphates, ATP is the primary energy source, acting to facilitate the synthesis of the others. ATP is made by three distinct types of phosphorylation – oxidative phosphorylation (in mitochondria), photophosphorylation (in chloroplasts of plants), and substrate-level phosphorylation (in enzymatically catalyzed reactions).

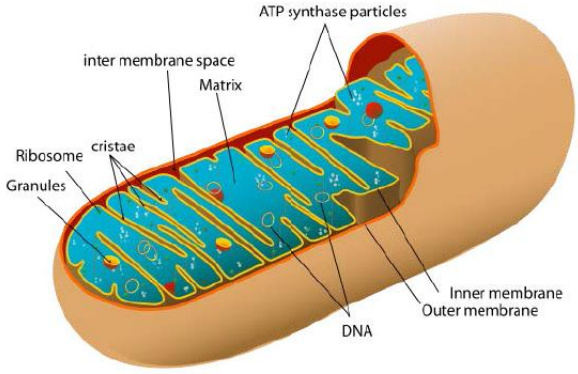


Figure 6.1.7: Mitochondrion

Gibbs free energy in biochemistry

ATP is generally considered the “storage currency” of cells (See also ‘Molecular Battery Backups for Muscles HERE). In order to understand how energy is captured, we must first understand Gibbs free energy and in doing so, we begin to see the role of energy in determining the direction chemical reactions take.

Gibbs free energy may be thought of as the energy available to do work in a thermodynamic system at constant temperature and pressure. Mathematically, the Gibbs free energy is given as:

$$G = H – TS \tag{6.1.1}$$

where H is the enthalpy, T is the temperature in Kelvin, and S is the entropy. At standard temperature and pressure, every system seeks to achieve a minimum of free energy. Thus, increasing entropy, S, will reduce Gibbs free energy. Similarly, if excess heat is available (reducing the enthalpy, H), the free energy can also be reduced.

Cells must work within the laws of thermodynamics, as noted, so all of their biochemical reactions, too, are ruled by these laws. Now we shall consider energy in the cell. The change in Gibbs free energy (ΔG) for a reaction is crucial, for it, and it alone, determines whether or not a reaction goes forward.

$$\Delta G = \Delta H – T\Delta S. \tag{6.1.2}$$

There are three cases

- ΔG < 0: the reaction proceeds as written
- ΔG = 0: the reaction is at equilibrium
- ΔG > 0: the reaction runs in reverse

For a reaction

$$a\,\mathrm{A} \rightleftharpoons b\,\mathrm{B} \tag{6.1.3}$$

(where ‘a’ and ‘b’ are integers and A and B are molecules) at pH 7, ΔG can be determined by the following equation,

$$\Delta G = \Delta G^{\circ\prime} + RT\ln\left(\frac{[B]^b}{[A]^a}\right) \tag{6.1.4}$$

For multiple substrate reactions, such as

$$a\,\mathrm{A} + c\,\mathrm{C} \rightleftharpoons b\,\mathrm{B} + d\,\mathrm{D} \tag{6.1.5}$$

$$\Delta G = \Delta G^{\circ\prime} + RT\ln\left(\frac{[B]^b[D]^d}{[A]^a[C]^c}\right) \tag{6.1.6}$$

The ΔG°' term is called the change in Standard Gibbs Free energy, which is the change in energy that occurs when all of the products and reactants are at standard conditions and the pH is 7.0. It is a constant for a given reaction.

In simple terms, we can collect all of the terms of the numerator together and call them {Products} and all of the terms of the denominator together and call them {Reactants},

$$\Delta G = \Delta G^{\circ\prime} + RT\ln\left(\frac{\{\text{Products}\}}{\{\text{Reactants}\}}\right) \tag{6.1.7}$$

For most biological systems, the temperature, T, is a constant for a given reaction. Since ΔG°' is also a constant for a given reaction, the ΔG is changed almost exclusively as the ratio of {Products}/{Reactants} changes.

Importance of ΔG°'

If one starts out at standard conditions, where everything except protons is at 1M, the RTln({Products}/{Reactants}) term is zero, so the ΔG°' term equals the ΔG, and the ΔG°' determines the direction the reaction will take (only under those conditions). This is why people say that a negative ΔG°' indicates an energetically favorable reaction, whereas a positive ΔG°' corresponds to an unfavorable one.

Increasing the ratio of {Products}/{Reactants} causes the value of the natural log (ln) term to become more positive (less negative), thus making the value of ΔG more positive. Conversely, as the ratio of {Products}/{Reactants} decreases, the value of the natural log term becomes less positive (more negative), thus making the value of ΔG more negative.

System response to stress

Intuitively, this makes sense and is consistent with Le Chatelier's Principle – a system responds to stress by acting to alleviate the stress. If we examine the ΔG for a reaction in a closed system, we see that it will always move to a value of zero (equilibrium), no matter whether it starts with a positive or negative value.

Another type of free energy available to cells is that generated by electrical potential. For example, mitochondria and chloroplasts partly use Coulombic energy (based on charge) from a proton gradient across their membranes to provide the necessary energy for the synthesis of ATP. Similar energies drive the transmission of nerve signals (sodium and potassium gradients) and the movement of some molecules in secondary active transport processes across membranes (e.g., H+ differential driving the movement of lactose). From the Gibbs free energy change equation,

$$\Delta G = \Delta H – T\Delta S \tag{6.1.8}$$

it should be noted that an increase in entropy will help contribute to a decrease in ΔG. This happens, for example when a large molecule is being broken into smaller pieces or when the rearrangement of a molecule increases the disorder of molecules around it. The latter situation arises in the hydrophobic effect, which helps drive the folding of proteins.

Chemical and electrical potential

It is said that absence makes the heart grow fonder. We won't tackle that philosophical issue here, but we will say that separation provides potential energy that cells can and do harvest. The lipid bilayer of cell and (in eukaryotic cells) organelle membranes provide the necessary barrier for separation.

Impermeable to most ions and polar compounds, biological membranes are essential for processes that generate cellular energy. Consider Figure 5.8. A lipid bilayer separates two solutions with different concentrations of a solute. There is a greater concentration of negative ions in the bottom and a greater concentration of positive ions on the top.

Whenever there is a difference in concentration of molecules across a membrane, there is said to be a concentration gradient across it. A difference in concentration of ions across a membrane also creates a charge (or electrical) gradient. Because there is a

difference in both the chemical concentration of the ions and in the charge on the two sides of the membrane, this is described as an electrochemical gradient (Figures 5.8 -5.10).

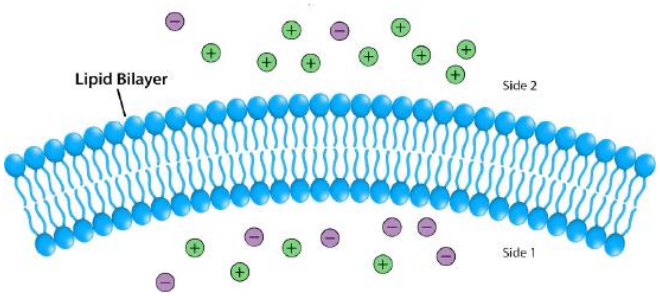


Figure 5.8: Differences in ion concentration across a membrane give rise to chemical and electrical gradients. Image by Pehr Jacobson

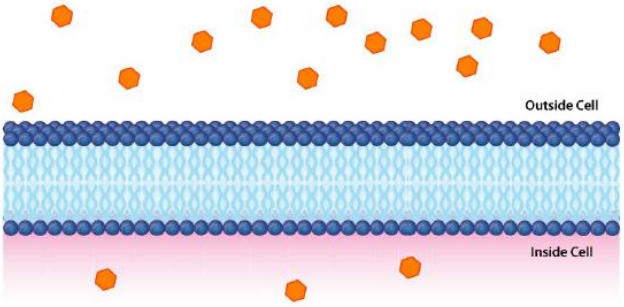


Figure **6.1.9**: *A chemical gradient. Image by Aleia Kim*

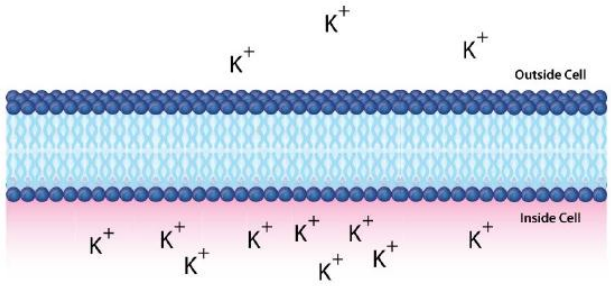


Figure **6.1.10**: An electrochemical gradient of potassium ions. Image by Aleia Kim

Potential energy

Such gradients function like batteries and contain potential energy. When the potential energy is harvested by cells, they can create ATP, transmit nerve signals, pump molecules across membranes, and more. It is important, therefore, to understand how to calculate the potential energy of electrochemical gradients.

First, we consider chemical (solute) gradients. In Figure 5.9, two concentrations of glucose are separated by a lipid bilayer. Let's assume C2 be the concentration of glucose inside the cell (bottom) and C1 be the glucose concentration outside (top). The Gibbs free energy associated with moving glucose in the direction of C2 (into the cell) is given by

$\Delta G = RT\ln[C2/C1]$

To move it in the direction of C1 (to the outside of the cell) the expression would be

$$\Delta G = RT\ln[C_1/C_2] \tag{6.1.9}$$

Since C2 is smaller than C1 (i.e., there are fewer glucose molecules inside the cell) then the ΔG is negative and diffusion would be favored into the cell, if the glucose could traverse the bilayer.

Conversely, if C2 was greater than C1 (more glucose was in the cell than outside) the ΔG would be positive, so movement in the direction of C2 would not be favored and instead the glucose would tend to move towards C1 , that is, out of the cell.

If C2 = C1, with the same concentration of glucose inside and outside, then the ΔG would be zero and there would be no net movement, as the system would be at equilibrium.

In the example above, we considered glucose, which is an uncharged molecule. When ions are involved, their charges must also be taken into consideration. Figure **6.1.1**0 depicts a similar situation across a lipid bilayer. In this case, a difference of concentration and charge exists. There are more positive charges inside the cell than outside.

Using C2 to indicate the concentration of materials inside the cell and C1 for the concentration outside the cell (as before), then the free energy for movement of an ion from top to bottom is given by the following equation

$$\Delta G = RT \ln[C_2/C_1] + ZF\Delta\psi \tag{6.1.10}$$

Note here that this equation must take into consideration both the concentration differences and the charge differences. Z refers to the charge of the transported species, F is the Faraday constant (96,485 Coulombs/mol), and Δψ is the electrical potential difference (voltage difference) across the membrane.

If we were to calculate the ΔG for movement of the potassium ion from top to bottom, it would be positive, since [C2/C1] is greater than 1 (making for a positive ln term), and the ZFΔψ is positive because positively charged ions (Z) are moving against a positive charge gradient given by Δψ (greater concentration at the target (bottom) than the starting point (top)). If we were to calculate the concentration of ions moving from bottom to top, then the ln term would be negative (C2<C1) and the ZFΔψ would be negative as well (Z=positive, but Δψ negative).

Reduction Potential

In discussing chemical potential, we must also consider reduction potential. Reduction potential measures the tendency of a chemical to be reduced by electrons. It is also designated by several other names/variables. These include redox potential, oxidation/reduction potential, ORP, pE, ε, E, and Eh.

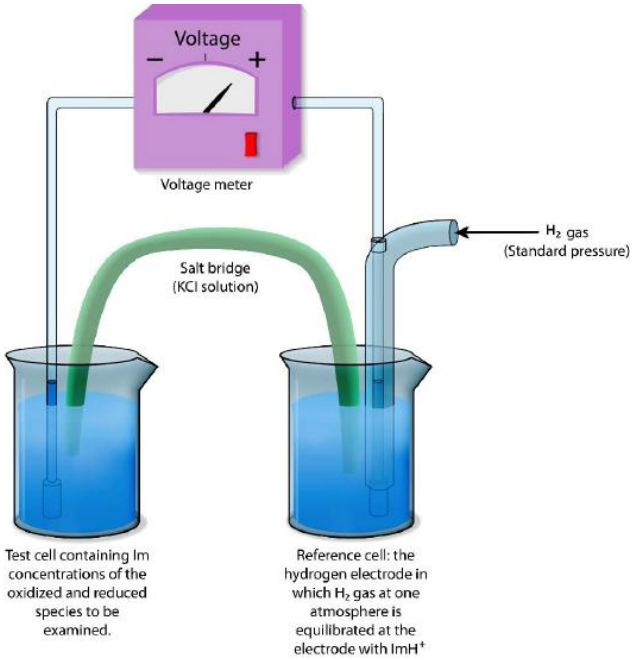


Figure **6.1.1**1: Reference electrode for measuring reduction potentials. Image by Pehr Jacobson

Reduction potential is measured in volts, or millivolts. A substance with a higher reduction potential will have a greater tendency to accept electrons and be reduced. If two substances are mixed in an aqueous solution, the one with the greater (more positive)

reduction potential will tend to take electrons away, thus being reduced, from the one with the lower reduction potential, which becomes oxidized.

Relative measures

Absolute reduction potentials are difficult to measure, so reduction potentials are typically defined relative to a reference electrode. In aqueous solutions, reduction potentials are measured as the potential difference between an inert sensing electrode (typically platinum) in contact with the test solution and a stable reference electrode (measured as a Standard Hydrogen Electrode: SHE) as shown in Figure **6.1.1**1. The standard of reference for measurement is the half-reaction

$$H^+ + e^- \rightarrow \frac{1}{2} H_2$$

The electrode where this reaction occurs (referred to as a half-cell) is given the value of E° (Standard Reduction Potential) of 0.00 volts. The hydrogen electrode is connected via an external circuit to another half cell containing a mixture of the reduced and oxidized species of another molecule (for example, Fe^{++} and Fe^{+++}) at 1M each and standard conditions of temperature (25°C) and pressure (1 atmosphere).

Direction and voltage measured

The direction and magnitude of electron movement is then measured. If the test mixture takes electrons from the hydrogen electrode, the sign of the voltage is positive and if the direction is reversed, the voltage is negative.

Thus, compounds which have greater affinity for electrons than hydrogen will register a positive voltage and negative voltages correspond to compounds with lesser affinity for electrons than hydrogen.

Movement of electrons

Under standard conditions, electrons will move from compounds generating lower voltages to ones generating higher (more positive) voltages. Just as the standard Gibbs free energy change is the Gibbs free energy change under standard conditions, so, too, is the standard reduction potential E° the reduction potential E under standard conditions.

The actual reduction potential of a half cell will vary with the concentration of each chemical species in the cell. The relationship between the reduction potential E and the standard reduction potential E° is given by the following equation (also called the Nernst equation)

$$E = E^\circ + (RT/nF)*\ln\left(\frac{[\text{reduced molecule}]}{[\text{oxidized molecule}]}\right)$$

where F is the Faraday constant (96,480 J/(Volts*moles), R is the gas constant (8.315 J/(moles*K), n is the number of moles of electrons being transferred, and T is the absolute temperature in Kelvin.

At 25°C, this equation becomes

$$E = E^\circ + (0.026V/n)*\ln\left(\frac{[\text{reduced molecule}]}{[\text{oxidized molecule}]}\right)$$

As for Gibbs free energy, it is useful to measure values at conditions found in cells. This means doing measurements at pH = 7, which differs from having all species at 1M.

Adjustment

Because of this adjustment, a slightly different standard reduction potential is defined and we designate it by E°', just as we defined a special standard Gibbs free energy change at pH 7 as ΔG°'.

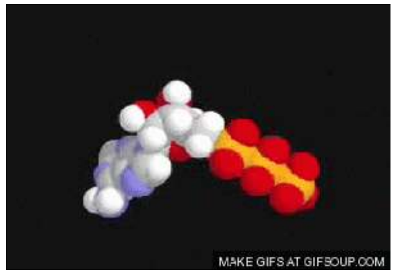
There is a relationship between the change in Gibbs free energy ΔG and the change in reduction potential (ΔE). It is

$$\Delta G = -nF\Delta E \tag{6.1.11}$$

Similarly, the relation between the change in standard Gibbs free energy and the change in standard reduction potential is

\]ΔG°' = -nFΔE°'\]

Energy in Triphosphates



Movie 5.1: ATP: The fuel of the cell

Formation of triphosphates, like ATP, is essential to meeting the cell's energy needs for synthesis, motion, and signaling. In a given day, an average human body makes and breaks down more than its weight in triphosphates. This is especially remarkable considering that there is only about 250 g of the molecule present in the body at any given time. Energy in ATP is released by hydrolysis of a phosphate from the molecule.

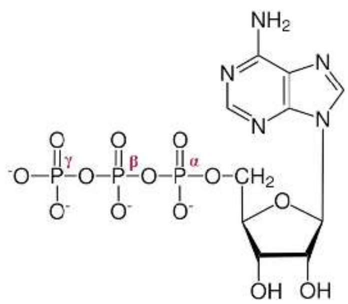


Figure 6.1.12 ATP showing α, β and γ phosphates

The three phosphates, starting with the one closest to the sugar are referred to as α, β, and γ (Figure 6.1.12). It is the γ phosphate that is cleaved in hydrolysis and the product is ADP. In a few reactions, the bond between the α and β is cleaved. When this happens, a pyrophosphate (β linked to γ) is released and AMP is produced. This latter reaction to produce AMP releases more energy ($\Delta G°'$ = -45.6 kJ/mol) than the first reaction which produces ADP ($\Delta G°'$ = -30.5 kJ/mol).

Since triphosphates are the "currency" that meet immediate needs of the cell, it is important to understand how triphosphates are made. There are three phosphorylation mechanisms – 1) substrate level; 2) oxidative; and 3) photophosphorylation. We consider them here individually.

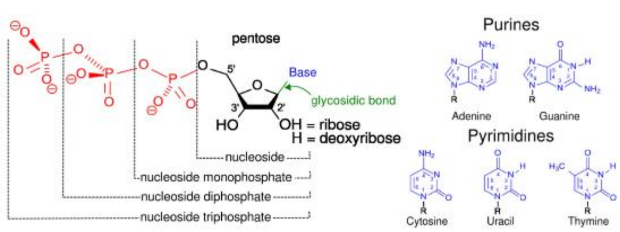


Figure 6.1.13: Nucleotides, nucleosides, and bases

Substrate level phosphorylation

The easiest type of phosphorylation to understand is that which occurs at the substrate level. This type of phosphorylation involves the direct synthesis of ATP from ADP and a high-energy intermediate, typically a phosphate-containing molecule. Substrate level phosphorylation is a relatively minor contributor to the total synthesis of triphosphates by cells. An example of substrate phosphorylation comes from glycolysis.

$$\text{Phosphoenolpyruvate (PEP)} + \text{ADP} \rightleftharpoons \text{Pyruvate} + \text{ATP}$$

This reaction has a very negative ΔG°' (-31.4 kJ/mol), indicating that the PEP contains more energy than ATP, thus tending to energetically favor ATP's synthesis. Other triphosphates can be made by substrate-level phosphorylation, as well. For example, GTP can be synthesized by the following citric acid cycle reaction.

$$\text{Succinyl-CoA + GDP + Pi } \rightleftharpoons \text{ Succinate + GTP + CoA-SH}$$

Triphosphates can be interchanged readily in substrate-level phosphorylations catalyzed by the enzyme Nucleoside Diphosphate Kinase (NDPK). A generalized form of the reactions catalyzed by this enzyme is as follows:

$$\text{XTP + YDP} \rightleftharpoons \text{XDP + YTP}$$

where X = adenosine, cytidine, uridine, thymidine, or guanosine and Y can be any of these as well. Further, XTP and YDP can be any of the deoxynucleotides as well.

Last, an unusual way of synthesizing ATP by substrate-level phosphorylation is via the reaction catalyzed by adenylate kinase

$$\text{2 ADP} \rightleftharpoons \text{ATP + AMP}$$

ATP source

This reaction is an important means of generating ATP when the cell doesn't have other sources of energy. Accumulation of AMP resulting from this reaction activates enzymes, such as phosphofructokinase, of glycolysis, which will catalyze reactions to give the cell additional, needed energy.

It is important to note that enzymes cannot make reactions happen that are energetically unfavorable. Enzymes speed reactions, but do not change their direction. Cells are thus bound by the rules of Gibbs free energy. So, how do energetically unfavorable reactions happen in a cell?

Reaction coupling

Reactions that are energetically unfavorable, can be made favorable by coupling them with the hydrolysis of ATP, a very energetically favorable reaction. There are numerous parallels in the "real world." The movement of automobiles is energetically unfavorable, but coupling movement of the automobile to oxidation of gasoline makes an unfavorable process favorable. Another approach to making an unfavorable reaction favorable is to manipulate the concentration of reactants and products. Consider the reaction below, which occurs in pyrimidine nucleotide metabolism:

$$\text{orotate + PRPP} \rightleftharpoons \text{OMP + PP}_\text{i}$$

The ΔG°' for this reaction is -0.8 kJ/mol, meaning that if one starts with equal concentrations of reactants and products, at equilibrium, there will be a small excess of products. In the cell, however, this reaction moves strongly to the right (ΔG = very negative). Given that the ΔG°' is very close to zero, a very negative ΔG can only occur if the concentrations of reactants and products are altered, since

$$\Delta G = \Delta G^{\circ\prime} + RT\ln\left(\frac{[\text{OMP}][\text{PP}_\text{i}]}{[\text{Orotate}][\text{PRPP}]}\right) \tag{6.1.12}$$

Manipulation is exactly what happens here. The key item whose concentration is adjusted in this reaction is the pyrophosphate (PPi). This is possible because cells contain an enzyme called pyrophosphorylase that catalyzes the following reaction

$$\text{PP}_\text{i} + \text{H}_2\text{O} \rightleftharpoons \text{2 P}_\text{i}$$

Hydrolysis of pyrophosphate is very energetically favored, causing the PPi produced in the reaction to be quickly hydrolyzed. As a result, the concentration of PPi in the cell is kept very low. A low concentration of a product (PPi) causes the natural log (ln) term of the orotate equation to become more negative, driving the ΔG term for the overall reaction to become much more negative.

Pushing and pulling

Reactions that yield pyrophosphate as a product are produced in the synthesis of DNA and RNA, as well as many other molecules. As shown in the previous example, this pyrophosphate is rapidly hydrolyzed, causing the overall reaction to move in the direction of pyrophosphate production. When reactants are removed/reduced in a metabolic reaction to decrease the concentration of a product, we say that the reaction is "pulled", to represent the increase in the forward reaction as a result of product depletion.

Pushing happens when reactants in a reaction are added/increased. This too has the effect of reducing the ΔG of a reaction and making it more favorable because the ratio of [Products]/[Reactants] is decreased with increasing [Reactants]. Pushing and pulling of reactions are additional tools for cells to overcome energy barriers, just like coupling energetically favorable processes to energetically unfavorable ones.

This page titled 6.1: Basics of Energy is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

6.2: Metabolism - Sugars

Glycolysis

Carbohydrates, whether synthesized by photosynthetic organisms, stored in cells as glycogen, or ingested by heterotrophs, must be broken down to obtain energy for the cell's activities as well as to synthesize other molecules required by the cell. Starch and glycogen, polymers of glucose, are the main energy storage forms of carbohydrates in plants and animals, respectively. To use these sources of energy, cells must first break down the polymers to yield glucose. The glucose is then taken up by cells through transporters in cell membranes. The metabolism of glucose, as well as other six carbon sugars (hexoses) begins with the catabolic pathway called glycolysis. In this pathway, sugars are oxidized and broken down into pyruvate molecules. The corresponding anabolic pathway by which glucose is synthesized is termed gluconeogenesis. Neither glycolysis nor gluconeogenesis is a major oxidative/reductive process, with one step in each one involving loss/gain of electrons, but the product of glycolysis, pyruvate, can be completely oxidized to carbon dioxide (Figure 6.2). Indeed, without production of pyruvate from glucose in glycolysis, a major energy source for the cell would not be available.

Glucose is the most abundant hexose in nature and is traditionally used to illustrate the reactions of glycolysis, but fructose (in the form of fructose-6- phosphate) is also readily metabolized, while galactose can easily be converted into glucose for catabolism in the pathway as well. The end metabolic products of glycolysis are two molecules of ATP, two molecules of NADH and two molecules of pyruvate (Figure 6.3), which, in turn, can be oxidized further in the citric acid cycle.

Entry points for glycolysis

Glucose and fructose are the sugar 'funnels' serving as entry points to the glycolytic pathway. Other sugars must be converted to either of these forms to be metabolized in glycolysis. Some pathways, including the Calvin

Figure 6.2 - Metabolic fates of glucose Image by Aleia Kim Your cells may have a mounting crisis Should they not go through glyco-lye-sis No glucose energy releases Until it's fractured into pieces

Figure 6.3 - Glycolysis and its Regulators Image by Ben Carson Cycle and the Pentose Phosphate Pathway (PPP) contain intermediates in common with glycolysis, so in that sense, almost any cellular sugar can be metabolized here.

Other pathways

Intermediates of glycolysis and gluconeogenesis that are common to other pathways include glucose-6-phosphate (PPP, glycogen metabolism), Fructose-6-phosphate (Calvin Cycle, PPP), Glyceraldehyde-3- phosphate (Calvin Cycle, PPP), dihydroxyacetone phosphate (PPP, glycerol metabolism, Calvin Cycle), 3- phosphoglycerate (Calvin Cycle, PPP), phosphoenolpyruvate (C4 plant metabolism, Calvin Cycle), and pyruvate (fermentation, acetyl-CoA genesis, amino acid metabolism). It is worth noting that glycerol from the breakdown of fat can readily be metabolized to dihydroxyacetone phosphate (DHAP) and thus enter the glycolysis pathway. It is the only part of a fat that is used in these pathways.

Reaction 1

Glucose gets a phosphate from ATP to make glucose-6-phosphate (G6P) in a reaction catalyzed by the enzyme hexokinase, a transferase enzyme.

Glucose + ATP ⇄ G6P + ADP + H+

Hexokinase is one of three regulated enzymes in glycolysis and is inhibited by one of the products of its action - G6P. Hexokinase has flexibility in its substrate binding and is able to phosphorylate a variety of hexoses, including fructose, mannose, and galactose.

Why phosphorylate glucose?

Phosphorylation of glucose serves two important purposes. First, the addition of a phosphate group to glucose effectively traps it in the cell, as G6P cannot diffuse across the lipid bilayer. Second, the reaction decreases the concentration of free glucose, favoring additional import of the molecule. G6P is a substrate for the pentose phosphate pathway and can also be converted to glucose-1-phosphate (G1P) for use in glycogen synthesis and galactose metabolism (Figure 6.5).

It is worth noting that the liver has an enzyme like hexokinase called glucokinase, which Figure 6.4 - Reaction #1 - Phosphorylation of glucose - catalyzed by hexokinase has a much higher Km (lower affinity) for glucose. This is important,

because the liver is a site of glucose synthesis (gluconeogenesis) where cellular concentrations of glucose can be relatively high. With a lower affinity glucose phosphorylating enzyme, glucose is not converted to G6P unless glucose concentrations get high, so the liver is able to release the glucose it makes into the bloodstream for the rest of the body to use.

Reaction 2

Next, G6P is converted to fructose-6-phosphate (F6P), in a reaction catalyzed by the enzyme

$$\mathrm{G_6P} \rightleftarrows \mathrm{F_6P} \tag{6.2.1}$$

The reaction has a low ΔG°' , so it is readily favorable in either direction with Figure 6.6 - Mechanism of conversion of G6P to F6P in reaction #2 Figure 6.5 - The centrality of glucose-6-phosphate in metabolism Image by Aleia Kim only slight changes in concentration of reactants.

Reaction 3

$$ceF6P + ATP \rightleftarrows F1,6BP + ADP + H+ \tag{6.2.2}$$

The second input of energy occurs when F6P gets another phosphate from ATP in a reaction catalyzed by the enzyme phosphofructokinase-1 (PFK-1 - another transferase) to make fructose-1,6- bisphosphate (F1,6BP). PFK-1 is a very important enzyme regulating glycolysis, with several allosteric activators and inhibitors (see HERE).

Like the hexokinase reaction the energy from ATP is needed to make the reaction energetically favorable. PFK-1 is the most important regulatory enzyme in the pathway and this reaction is the ratelimiting step. It is also one of three essentially irreversible reactions in glycolysis.

A variant enzyme found in plants and some bacterial uses pyrophosphate rather than ATP as the energy source and due to the lower energy input from hydrolysis of the pyrophosphate, that reaction is reversible.

Reaction 4

$$\mathrm{F_{1,6}BP} \rightleftarrows \mathrm{D{-}GLYAL_3P + DHAP} \tag{6.2.3}$$

With the glycolysis pump thus primed, the pathway proceeds to split the F1,6BP into two 3-carbon intermediates. This reaction catalyzed by the lyase known as aldolase is energetically a “hump” to overcome in the glycolysis direction (ΔG°' = +24 kJ/mol Figure 6.7 - Reaction #3 - Conversion of F6P to F1,6BP by PFK Wikipedia Figure 6.8 - Reaction #4 - Breakdown of F1,6BP into GLYAL3P (left) and DHAP (right) by aldolase °K) so to get over the energy hump, cells must increase the concentration the reactant (F1,6BP) and decrease the concentration of the products, which are D-glyceraldehyde- 3-phosphate (D-GLYAL3P) and dihydroxyacetone phosphate (DHAP).

A novel scheme facilitates decreasing concentration of the products (see below). Aldolases cut the ketose ring by two different mechanisms and these enzymes are grouped as Class I (in animals and plants) and Class II (in fungi and bacteria).

Reaction 5

$$\mathrm{DHAP} \rightleftarrows \mathrm{D{-}GLYAL_3P} \tag{6.2.4}$$

In the next step, DHAP is converted to DGLYAL3P in a reaction catalyzed by the enzyme triosephosphate isomerase. At this point, the six carbon glucose molecule has been broken down to two units of three carbons each - D-GLYAL3P. From this point forward each reaction of glycolysis contains two of each molecule. Reaction #5 is fairly readily reversible in cells.

The enzyme is of note because it is one example of a “perfect enzyme.” Enzymes in this category have very high ratios of Kcat/Km that approach a theoretical maximum limited only by the diffusion of substrate into the active site of the enzyme. The apparent reason for the enzyme evolving in this way is that the mechanism of the reaction produces an unstable, toxic intermediate (Figure 6.9). With the reaction proceeding as rapidly as it does, there is less chance of the intermediate escaping and causing damage in the cell.

Reaction 6

$$\mathrm{D{-}GLYAL_3P + NAD^+ + Pi\ D\text{-}1,3\,BPG + NADH + H^+} \tag{6.2.5}$$

Figure 6.9 - Reaction #5 - Triose phosphate isomerase with unstable, toxic intermediate (methyl glyoxal) Image by Ben Carson

In this reaction, D-GLYAL3P is oxidized in the only oxidation step of glycolysis catalyzed by the enzyme glyceraldehyde-3-phosphate dehydrogenase, an oxidoreductase. The aldehyde in this reaction is oxidized, then linked to a phosphate to make an ester - D-1,3-bisphospho-glycerate (D- 1,3BPG). Electrons from the oxidation are donated to NAD+, creating NADH.

NAD+ is a critical constituent in this reaction and is the reason that cells need a fermentation option at the end of the pathway (see below).

Note here that ATP energy was not required to put the phosphate onto the oxidized D-GLYAL3P. The reason for this is because the energy provided by the oxidation reaction is sufficient for adding the phosphate.

Reaction 7

$$\text{D-1,3 BPG} + \text{ADP} \rightleftarrows \text{3 PG} + \text{ATP} \tag{6.2.6}$$

The two phosphates in the tiny 1,3BPG molecule repel each other and give the molecule high potential energy. This energy is utilized by the enzyme phosphoglycerate kinase (another transferase) to phosphorylate ADP and make ATP, as well as the product, 3-phosphoglycerate (3-PG). This is an example of a substrate-level phosphorylation. Such mechanisms for making ATP require an intermediate with a high enough energy to phosphorylate ADP to make ATP. Figure 6.10 - Reaction #6 - Oxidation of GLYAL3P, catalyzed by glyceraldehyde-3-phosphate dehydrogenase Figure 6.11 - Reaction #7 - Substrate-level Phosphorylation by 1,3-BPG

Though there are a few substrate level phosphorylations in cells (including another one at the end of glycolysis), the vast major of ATP is made by oxidative phosphorylation in the mitochondria (in animals). In addition to oxidative phosphorylation, plants also make ATP by photophosphorylation in their chloroplasts. Since there are two 1,3 BPGs produced for every glucose, the two ATPs produced in this reaction replenish the two ATPs used to start the cycle and the net ATP count at this point of the pathway is zero.

Reaction 8

$$\text{3-PG} \rightleftarrows \text{2-PG} \tag{6.2.7}$$

Conversion of the 3-PG intermediate to 2-PG (2- phosphoglycerate) occurs by an important mechanism. An intermediate in this readily reversible reaction (catalyzed by phosphoglycerate mutase - a mutase enzyme) is 2,3-BPG. This intermediate, which is stable, is released with low frequency by the enzyme instead of being con- Figure 6.13 - Two routes to formation of 2,3-BPG Figure 6.14 - 2,3- Bisphosphoglycerate (2,3-BPG) Figure 6.12 - Reaction #8 - Conversion of 3-PG to 2-PG verted to 2-PG. 2,3BPG is important because it binds to hemoglobin and stimulates release of oxygen. The molecule can also be made from 1,3-BPG as a product of a reaction catalyzed by bisphophglycerate mutase (Figure 6.13). Cells which are metabolizing glucose rapidly release more 2,3-BPG and, as a result, get more oxygen, supporting their needs. Notably, cells which are metabolizing rapidly are using oxygen more rapidly and are more likely to be deficient in it.

Reaction 9

$$\text{2-PG} \rightleftarrows \text{PEP} + \text{H}_2\text{O} \tag{6.2.8}$$

2-PG is converted by enolase (a lyase) to phosphoenolpyruvate (PEP) by removal of water, creating a very high energy intermediate. The reaction is readily reversible, but with PEP, the cell has one of its highest energy molecules and that is important for the next reaction.

Reaction 10

$$\text{PEP} + \text{ADP} + \text{H}^+ \rightleftarrows \text{PYR} + \text{ATP} \tag{6.2.9}$$

Conversion of PEP to pyruvate by pyruvate kinase is the second substrate level phosphorylation of glycolysis, creating ATP. This reaction is what some refer to as the “Big Bang” of glycolysis because there is almost enough energy in PEP to stimulate production of a second ATP ($\Delta G^{\circ\prime}$ = 31.6 kJ/ mol), but it is not used. Consequently, this energy is lost as heat. If you wonder why you get hot when you exercise, the heat produced in the breakdown of glucose is a prime contributor and the pyruvate kinase reaction is a major source. Figure 6.16 - Reaction #10 - The big bang - PEP phosphorylates ADP with a lot of energy to spare Wikipedia Figure 6.15 - Reaction #9 - Enolase-catalyzed removal of water Wikipedia

Pyruvate kinase is the third and last enzyme of glycolysis that is regulated (see below). The primary reason this is the case is to be able to prevent this reaction from occurring when cells are making PEP while going through gluconeogenesis (see more HERE).

Catabolism of other sugars

Though glycolysis is a pathway focused on the metabolism of glucose and fructose, the fact that other sugars can be readily metabolized into glucose means that glycolysis can be used for extracting energy from them as well. Galactose is a good example. It is commonly produced in the produced in the body as a result of hydrolysis of lactose, catalyzed by the enzyme known as lactase (Figure 6.17). Deficiency of lactase is the cause of lactose intolerance.

Galactose begins preparation for entry into glycolysis by being converted to galactose-1- phosphate (catalyzed by galactokinase - Figure 6.18). Galactose-1-phosphate swaps with glucose-1-phosphate from UDP-glucose to make UDP-galactose (Figure 6.19). An epimerase converts UDPgalactose back to UDP-glucose and the cycle is complete. Each turn of the cycle thus takes in one galactose-1-phosphate and releases one glucose-1-phosphate.

Deficiency of galactose conversion enzymes results in accumulation of galactose (from breakdown of lactose). Excess galactose is converted to galactitol, a sugar alcohol. Galactitol in the human eye lens causes it to absorb water and this may be a factor in formation of cataracts.Figure 6.17 - Breakdown of lactose to glucose and galactose by lactase Image by Pehr Jacobson Figure 6.18 - Galactokinase Reaction Image by Penelope Irving Free fructose can also enter glycolysis by two mechanisms. First, it can be phosphorylated to fructose-6-phosphate by hexokinase. A more interesting alternate entry point is that shown in Figure 6.20. Phosphorylation of fructose by fructokinase produces fructose-1-phosphate and cleavage of that by fructose-1- phosphate aldolase yields DHAP and glyceraldehyde.

Phosphorylation of glyceraldehyde by triose kinase yields GLYAL3P. This alternative entry means for fructose may have important implications because DHAP and GLYAL3P are introduced into the glycolysis pathway while bypassing PFK-1 regulation. Some have proposed this may be important when considering metabolism of high fructose corn syrup, since it forces production of pyruvate, a precursor of acetyl-CoA, which is itself a precursor of fatty acids when ATP levels are high.

Mannose metabolism

Mannose can also be metabolized in glycolysis. In this case, it enters via fructose by the following two-step process - 1) phosphoryla- Figure 6.19 - Conversion of galactose-1-phosphate into glucose-6-phosphate Image by Aleia Kim tion by hexokinase to make mannose-6- phosphate followed by its conversion to fructose-6-phosphate, catalyzed by phosphomannoisomerase (Figure 6.21).

Glycerol metabolism

Glycerol is an important molecule for the synthesis of fats, glycerophospholipids, and other membrane lipids. Most commonly it is made into glycerol-3- phosphate (Figure 6.22) and the glycolysis/gluconeogenesis pathways are important both for producing the compound and for metabolizing it. The relevant intermediate in these pathways both for producing and for using glycerol-3-phosphate is DHAP. The enzyme glycerol-3-phosphate dehydrogenase reversibly converts glycerol-3- phosphate into DHAP (Figure 6.22).

This reaction, which is an oxidation, transfers electrons to NAD+ to produce NADH. In the reverse reaction, production of glycerol-3- phosphate from DHAP, of course, requires electrons from NADH for the reduction. Both glycolysis and gluconeogenesis are sources DHAP, meaning when the cell needs glycerol- 3-phosphate that it can use sugars (glucose, fructose, mannose, or galactose) as sources in glycolysis. For gluconeogenesis, sources include pyruvate, alanine and Figure 6.20 - Entry of fructose into glycolysis, bypassing PFK-1 Image by Penelope Irving Figure 6.21 - Entry of other sugars into glycolysis Image by Penelope Irving lactate (both can easily be made into pyruvate), oxaloacetate, aspartic acid (which can be made into oxaloacetate by transamination), and others. All of the intermediates of the citric acid cycle (and glyoxylate cycle) can be converted ultimately to oxaloacetate, which is a gluconeogenesis intermediate, as well.

It is worth noting that animals are unable to use fatty acids as materials for gluconeogenesis in net amounts, but they can, in fact, use glycerol in both glycolysis and gluconeogenesis. It is the only part of the fat molecule that can be so used.

Pyruvate metabolism

As noted, pyruvate produced in glycolysis can be oxidized to acetyl-CoA, which is itself oxidized in the citric acid cycle to carbon dioxide. That is not the only metabolic fate of pyruvate, though (Figure 6.23).

Pyruvate is a “starting” point for gluconeogenesis, being converted to oxaloacetate in the mitochondrion in the first step. Pyruvate in animals can also be reduced to lactate by adding electrons from NADH (Figure 6.24). This reaction produces NAD+ and is critical for generating the latter molecule to keep the glyceraldehyde-3-phosphate dehydrogenase reaction of glycolysis (reaction #6) going under conditions when there is no oxygen.

This is because oxygen is necessary for the electron transport system (ETS) to operate and it performs the important function of converting NADH back to NAD+. When the ETS is running, NADH donates electrons to Complex I and is oxidized to NAD+ in the process, generating the intermediate needed for oxidizing GLYAL-3P. In the absence of oxygen, however, NADH cannot be converted to Figure 6.22 - Reactions in glycerol metabolism Image by Penelope Irving NAD+ by the ETS, so an alternative means of making NAD+ is necessary for keeping glycolysis running under low oxygen conditions (fermentation).

Bacteria and yeast generate NAD+ under oxygen deprived conditions by doing fermentation in a different way (Figure 6.25). They use NADH-requiring reactions that regenerate NAD+ while producing ethanol from pyruvate instead of making lactate. Thus, fermentation of pyruvate is essential to keep glycolysis operating when oxygen is limiting. It is also for these reasons that brewing of beer (using yeast) involves depletion of oxygen and muscles low in oxygen produce lactic acid (animals).

Pyruvate is a precursor of alanine which can be easily synthesized by transfer of a nitrogen from an amine donor, such as glutamic acid. Pyruvate can also be converted into oxaloacetate by carboxylation in the process of gluconeogenesis (see below).

The enzymes involved in pyruvate metabolism include pyruvate dehydrogenase (makes acetyl-CoA), lactate dehydrogenase (makes lactate), transaminases (make alanine), pyruvate carboxylase (makes ox- Figure 6.23 - Pyruvate metabolism. When oxygen is absent, pyruvate is converted to lactate (animals) or ethanol (bacteria and yeast). When oxygen is present, pyruvate is converted to acetyl-CoA. Not shown - Pyruvate transamination to alanine or carboxylation to form oxaloacetate. aloacetate), and pyruvate decarboxylase (a part of pyruvate dehydrogenase that makes acetaldehyde in bacteria and yeast).

Catalytic action and regulation of the pyruvate dehydrogenase complex is discussed in the section on the citric acid cycle (HERE).

Gluconeogenesis

The anabolic counterpart to glycolysis is gluconeogenesis (Figure 6.26), which occurs mostly in the cells of the liver and kidney and virtually no other cells in the body. In seven of the eleven reactions of gluconeogenesis (starting from pyruvate), the same enzymes are used as in glycolysis, but the reaction directions are reversed. Notably, the ΔG values of these reactions in the cell are typically near zero, meaning their direction can be readily controlled by changing substrate and product concentrations by small amounts.

The three regulated enzymes of glycolysis all catalyze reactions whose cellular ΔG values are not close to zero, making manipulation of reaction direction for their reac- Figure 6.24 - Formation of lactate in animal fermentation produces NAD+ for G3PDH Image by Ben Carson Figure 6.25 - Formation of ethanol in microbial fermentation produces NAD+ for G3PDH Image by Ben Carson tions non-trivial. Consequently, cells employ “work-around” reactions catalyzed by four different enzymes to favor gluconeogenesis, when appropriate.

Bypassing pyruvate kinase

Two of the enzymes (pyruvate carboxylase and PEP carboxykinase - PEPCK) catalyze reactions that bypass pyruvate kinase. F1,6BPase bypasses PFK-1 and G6Pase bypasses hexokinase. Notably, pyruvate carboxylase and G6Pase are found in the mitochondria and endoplasmic reticulum, respectively, whereas the other two are found in the cytoplasm along with all of the enzymes of glycolysis.

Biotin An important coenzyme used by pyruvate carboxylase is biotin (Figure 6.27). Biotin is commonly used by carboxylases to carry CO2 to incorporate into the substrate.

Also known as vitamin H, biotin is a water soluble B vitamin (B7) needed for many metabolic processes, including fatty acid synthesis, gluconeogenesis, and amino acid metabolism. Deficiency of the vitamin is rare, since it is readily produced by gut Gluconeogenesis and glycolysis. Only the enzymes differing in gluconeogenesis are shown Image by Aleia Kim teria. There are many claims of advantages of taking biotin supplements, but there is no strong indication of benefits in most cases. Deficiencies are associated with inborn genetic errors, alcoholism, burn patients, and people who have had a gastrectomy. Some pregnant and lactating women may have reduced levels due to increased biotin catabolism.

Reciprocal regulation

All of the enzymes of glycolysis and nine of the eleven enzymes of gluconeogenesis are all in the cytoplasm, necessitating a coordinated means of controlling them. Cells generally need to minimize the extent to which paired anabolic and catabolic pathways are occurring simultaneously, lest they produce a futile cycle, resulting in wasted energy with no tangible product except heat. The mechanisms of controlling these pathways have opposite effects on catabolic and anabolic processes. This method of control is called reciprocal regulation (see above).

Reciprocal regulation is a coordinated means of simultaneously controlling metabolic pathways that do opposite things. In reciprocal regulation, a single molecule (allosteric regulation) or a single covalent modification (phosphorylation/dephosphorylation,

Allosteric Regulation of Glycolysis & Gluconeogenesis

Reciprocal Regulation

AMP - Activates PFK-1, Inhibits F1,6BPase

F2,6BP - Activates PFK-1, Inhibits F1,6BPase

Citrate - Activates PFK-1, Inhibits F1,6BPase

Glycolysis Only

ATP - Inhibits PFK-1 and Pyruvate Kinase

Alanine - Inhibits Pyruvate Kinase

Gluconeogenesis Only

ADP - Inhibits Pyruvate Carboxylase and PEPCK

Acetyl-CoA - Activates Pyruvate Carboxylase

Figure 6.27 - Biotin carrying carbon dioxide (red) Wikipedia for example) has opposite effects on the different pathways.

Reciprocal allosteric effects For example, in glycolysis, the enzyme known as phosphofructokinase (PFK-1) is allosterically activated by AMP and a molecule known as F2,6BP (Figure 6.28). The corresponding enzyme from gluconeogenesis catalyzing a reversal of the glycolysis reaction is known as F1,6BPase. F1,6BPase is inhibited by both AMP and F2,6BP.

Reciprocal covalent effects

In glycogen metabolism, the enzymes phosphorylase kinase and glycogen phosphorylase catalyze reactions important for the breakdown of glycogen. The enzyme glycogen synthase catalyzes the synthesis of glyco- Directional velocity Inverts with reciprocity If glycolysis is flowing Glucose synthesis awaits But when the latter is a-going Sugar breakdown then abates Figure 6.28 - Regulation of glycolysis (orange path) and gluconeogenesis (black path) Image by Aleia Kim gen. Each of these enzymes is, at least partly, regulated by attachment and removal of phosphate.

Phosphorylation of phosphorylase kinase and glycogen phosphorylase has the effect of making them more active, whereas phosphorylation of glycogen synthase makes it less active. Conversely, dephosphorylation has the reverse effects on these enzymes - phosphorylase kinase and glycogen phosphorylase become less active and glycogen synthase becomes more active.

Simple and efficient

The advantage of reciprocal regulation schemes is that they are very efficient. It doesn't take separate molecules or separate treatments to control two pathways simultaneously. Further, its simplicity ensures that when one pathway is turned on, the other is turned off.

This is especially important with catabolic/ anabolic regulation, because having both pathways going on simultaneously in a cell is not very productive, leading only to production of heat in a futile cycle. A simple futile cycle is shown on Figure 6.29. If unregulated, the cyclic pathway in the figure (shown in black) will make ATP in creating pyruvate from PEP and will use ATP to make oxaloacetate from pyruvate.

It will also use GTP to make PEP from oxaloacetate. Thus, each turn of the cycle will make one ATP, use one ATP and use one GTP for a net loss of energy. The process will start with pyruvate and end with pyruvate, so there is no net production of molecules. (see HERE for one physiological use of a futile cycle).

Specific gluconeogenesis controls

Besides reciprocal regulation, other mechanisms help control gluconeogenesis. First, PEPCK is controlled largely at the level of synthesis. Overexpression of PEPCK (stimulated by glucagon, glucocorticoid hormones, and cAMP and inhibited by insulin) produces symptoms of diabetes.

Pyruvate carboxylase is sequestered in the mitochondrion (one means of regulation) Figure 6.29 - A simple futile cycle - follow the black lines Image by Aleia Kim Interactive Learning Module HERE and is sensitive to acetyl-CoA, which is an allosteric activator. Acetyl-CoA concentrations increase as the citric acid cycle activity decreases. Glucose-6- phosphatase is present in low concentrations in many tissues, but is found most abundantly and importantly in the major gluconeogenic organs – the liver and kidney cortex.

Specific glycolysis controls

Control of glycolysis and gluconeogenesis is unusual for metabolic pathways, in that regulation occurs at multiple points. For glycolysis, this involves three enzymes:

1. Hexokinase (Glucose ⇄ G6P)

2. Phosphofructokinase-1 (F6P ⇄ F1,6BP)

3. Pyruvate kinase (PEP ⇄ Pyruvate).

Regulation of hexokinase is the simplest of these. The enzyme is unusual in being inhibited by its product, glucose-6-phosphate. This ensures when glycolysis is slowing down hexokinase is also slowing down to reduce feeding the pathway.

Pyruvate kinase

It might also seem odd that pyruvate kinase, the last enzyme in the pathway, is regulated (Figure 6.30), but the reason is simple. Pyruvate kinase catalyzes the most energetically rich reaction of glycolysis. The reaction is favored so strongly in the forward direction that cells must do a ‘two-step’ around it in the reverse direction when making glucose in the gluconeogenesis pathway. In other words, it takes two enzymes, two reactions, and two triphosphates (ATP and GTP) to go from one pyruvate back to one PEP in gluconeogenesis. When cells are needing to make glu- igure 6.30 - Regulation of pyruvate kinase For cells a glucose cycling’s cost Is energy in reams Four ATPs each time is lost From breaking/making schemes So use for metabolic heat To make it warm inside your feet Else it’s of no utility To practice such futility cose, they can’t be sidetracked by having the PEP they have made in gluconeogenesis be converted directly back to pyruvate by pyruvate kinase. Consequently, pyruvate kinase must be inhibited during gluconeogenesis or a futile cycle will occur and no glucose will be made.

Another interesting control mechanism called feedforward activation involves pyruvate kinase. Pyruvate kinase is activated allosterically by the glycolysis intermediate, F1,6BP. This molecule is a product of the PFK-1 reaction and a substrate for the aldolase reaction.

Reactions pulled

As noted above, the aldolase reaction is energetically unfavorable (high positive ΔG°’), thus allowing F1,6BP to accumulate. When this happens, some of the excess F1,6BP binds to pyruvate kinase, which activates and jump- Figure 6.31 - Regulation of Synthesis and Breakdown of F2,6BP Image by Penelope Irving starts the conversion of PEP to pyruvate. The resulting drop in PEP levels has the effect of “pulling” on the reactions preceding pyruvate kinase. As a consequence, the concentrations of GLYAL3P and DHAP fall, helping to pull the aldolase reaction forward.

PFK-1 regulation

PFK-1 has a complex regulation scheme. First, it is reciprocally regulated (relative to F1,6BPase) by three molecules. F2,6BP activates PFK-1 and inhibits F1,6BPase. PFK-1 is also allosterically activated by AMP, whereas F1,6BPase is inhibited. On the other hand, citrate inhibits PFK-1, but activates F1,6BPase.

PFK-1 is also inhibited by ATP and is exquisitely sensitive to proton concentration, easily losing activity when the pH drops only slightly. PFK- 1's inhibition by ATP is noteworthy and odd at first glance because ATP is also a substrate whose increasing concentration should favor the reaction instead of inhibit it. The root of this conundrum is that PFK-1 has two ATP binding sites - one at an allosteric site that binds ATP relatively inefficiently and one that the active site that binds ATP with high affinity. Thus, only when ATP concentration is high is binding at the allosteric site favored and only then can ATP turn off the enzyme.

F2,6BP regulation

Regulation of PFK-1 by F2,6BP is simple at the PFK-1 level, but more complicated at the level of synthesis of F2,6BP. Despite having a name sounding like a glycolysis/ gluconeogenesis intermediate (F1,6BP), F2,6BP is not an intermediate in either pathway. Instead, it is made from fructose-6-phosphate and ATP by the enzyme known as phosphofructokinase-2 (PFK- 2 - Figure 6.31).

Cori cycle

With respect to energy, the liver and muscles act complementarily. The liver is the major or- Figure 6.32 - The Cori cycle Image by Aleia Kim gan in the body for the synthesis of glucose. Muscles are major users of glucose to make ATP. Actively exercising muscles use oxygen faster than the blood can deliver it. As a consequence, the muscles go anaerobic and produce lactate. This lactate is of no use to muscle cells, so they dump it into the blood. Lactate travels in the blood to the liver, which takes it up and reoxidizes it back to pyruvate, catalyzed by the enzyme lactate dehydrogenase (Figure 6.32).

Pyruvate in the liver is then converted to glucose by gluconeogenesis. The glucose thus made by the liver is dumped into the bloodstream where it is taken up by muscles and used for energy, completing the important intercellular pathway known as the Cori cycle.

Glucose alanine cycle

The glucose alanine cycle (also known as the Cahill Cycle), has been described as the amine equivalent of the Cori cycle (Figure 6.33). The Cori cycle, of course, exports lac- Figure 6.33 - Overlap between the Cori cycle and the glucose alanine cycle tate from muscles (when oxygen is limiting) to the liver via the bloodstream. The liver, in turn, converts lactate to glucose, which it ships back to the muscles via the bloodstream. The Cori Cycle is an essential source of glucose energy for muscles during periods of exercise when oxygen is used faster than it can be delivered.

In the glucose-alanine cycle, cells are generating toxic amines and must export them. This is accomplished by transaminating pyruvate (the product of glycolysis) to produce the amino acid alanine.

The glucose-alanine process requires the enzyme alanine aminotransferase, which is found in muscles, liver, and intestines. Alanine is exported in the process to the blood and picked up by the liver, which deaminates it to release the amine for synthesis of urea and excretion. The pyruvate left over after the transamination is a substrate for gluconeogenesis. Glucose produced in the liver is then exported to the blood for use by cells, thus completing the cycle.

Polysaccharide metabolism

Sugars are metabolized rapidly in the body and that is one of the primary reasons they are used. Managing levels of glucose in the body is very important - too much leads to complications related to diabetes and too little gives rise to hypoglycemia (low blood sugar). Sugars in the body are maintained by three processes - 1) diet; 2) synthesis (gluconeogenesis); and 3) storage. The storage forms of sugars are, of course, the polysaccharides and their metabolism is our next topic of discussion.

Amylose and amylopectin

The energy needs of a plant are much less dynamic than those of animals. Muscular contraction, nervous systems, and information processing in the brain require large amounts of quick energy. Because of this, the polysaccharides stored in plants are somewhat less complicated than those of animals. Plants store glucose for energy in the form of amylose (Figure 6.34 and see HERE) and amylopectin and for structural integrity in the form of cellulose (see HERE). These structures differ in that cellulose contains glucose units solely joined by β-1,4 bonds, whereas amylose has only α-1,4 bonds and amylopectin has α-1,4 and α-1,6 bonds. Figure 6.34 Amylose, a polymer of glucose in plants

Glycogen

Animals store glucose primarily in liver and muscle in the form of a compound related to amylopectin known as glycogen. The structural differences between glycogen and amylopectin are solely due to the frequency of the α-1,6 branches of glucoses. In glycogen they occur about every 10 residues instead of every 30-50, as in amylopectin (Figure 6.35).

Glycogen provides an additional source of glucose besides that produced via gluconeogenesis. Because glycogen contains so many glucoses, it acts like a battery backup for the body, providing a quick source of glucose when needed and providing a place to store excess glucose when glucose concentrations in the blood rise.

The branching of glycogen is an important feature of the molecule metabolically as well. Since glycogen is broken down from the "ends" of the molecule, more branches translate to more ends, and more glucose that can be released at once.

Just as in gluconeogenesis, the cell has a separate mechanism for glycogen synthesis that is distinct from glycogen breakdown. As noted previously, this allows the cell to separately control the reactions, avoiding futile cycles, and enabling a process to occur efficiently (synthesis of glycogen) that would not occur if Figure 6.35 - Glycogen Structure - α-1,4 links with α-1,6 branches every 7-10 residues it were simply the reversal of glycogen breakdown.

Glycogen breakdown

Breakdown of glycogen involves 1) release of glucose-1-phosphate (G1P), 2) rearranging the remaining glycogen (as necessary) to permit continued breakdown, and 3) conversion of G1P to G6P for further metabolism. G6P can be 1) used in glycolysis, 2) converted to glucose by gluconeogenesis, or 3) oxidized in the pentose phosphate pathway.

Glycogen phosphorylase (sometimes simply called phosphorylase) catalyzes breakdown of glycogen into glucose-1- Phosphate (G1P - Figure 6.36). The reaction that produces G1P from glycogen is a phosphorolysis, not a hydrolysis reaction. The distinction is that hydrolysis reactions use water to cleave bigger molecules into smaller ones, but phosphorolysis reactions use phosphate instead for the same purpose. Note that the phosphate is just that - it does NOT come from ATP. Since ATP is not used to put phosphate on G1P, the reaction saves the cell energy.

Glycogen debranching enzyme

Glycogen phosphorylase will only act on nonreducing ends of a glycogen chain that are at least 5 glucoses away from a branch point. A second enzyme, Glycogen Debranching Enzyme (GDE) (also called debranching enzyme), is therefore needed to convert α (1-6) branches to α (1-4) branches. GDE acts on glycogen branches that have reached their limit of phosphorylysis with glycogen phosphorylase. Figure 6.36 - Breaking of α-1,4 bonds of glycogen by glycogen phosphorylase Image by Aleia Kim Interactive Learning Module HERE

GDE acts to transfer a trisaccharide from an α-1,6 branch onto an adjacent α-1,4 branch, leaving a single glucose at the 1,6 branch. Note that the enzyme also catalyzes the hydrolysis of the remaining glucose at the 1,6 branch point (Figure 6.37). Thus, the breakdown products from glycogen are G1P and glucose (mostly G1P). Glucose can, of course, be converted to Glucose-6-Phosphate (G6P) as the first step in glycolysis by either hexokinase or glucokinase.

G1P can be converted to G6P by action of an enzyme called phosphoglucomutase. This reaction is readily reversible, allowing G6P and G1P to be interconverted as the concentration of one or the other increases. This is important, because phosphoglucomutase is needed to form G1P for glycogen synthesis.

Regulation of glycogen metabolism

Regulation of glycogen metabolism is complex, occurring both allosterically and via hormone-receptor controlled events that result in protein phosphorylation or dephosphorylation. In order to avoid a futile cycle of glycogen synthesis and breakdown simultaneously, cells have evolved an elaborate set of controls that ensure only one pathway is primarily active at a time.

Regulation of glycogen metabolism is managed by the enzymes glycogen phosphorylase and glycogen synthase. Glycogen phosphorylase is regulated by both allosteric factors (ATP, G6P, AMP, and glucose) and by covalent modification (phosphorylation / dephosphorylation). Its regulation is consistent with the energy needs of the cell. High energy molecules (ATP, G6P, glucose) al- Figure 6.37 - Catalytic activity of debranching enzyme losterically inhibit glycogen phosphorylase, while the low energy molecule AMP allosterically activates it.

GPa/GPb allosteric regulation

Glycogen phosphorylase exists in two different covalent forms – one form with phosphate (called GPa here) and one form lacking phosphate (GPb here). GPb is converted to GPa by phosphorylation by an enzyme known as phosphorylase kinase. GPa and GPb can each exist in an 'R' state and a 'T' state (Figure 6.38). For both GPa and GPb, the R state is the more active form of the enzyme. GPa's negative allosteric effector (glucose) is usually not abundant in cells, so GPa does not flip into the T state often. There is no positive allosteric effector of GPa. When glucose is absent, GPa automatically flips into the R (more active) state (Figure 6.39). It is for this reason that people tend to think of GPa as being the more active covalent form of the enzyme.

GPb can convert from the GPb T state to the GPb R state by binding AMP. Unless a cell is low in energy, AMP concentration is low. Thus GPb is not converted Figure 6.38 - Glycogen phosphorylase regulation - covalent (horizontal) and allosteric (vertical) Image by Aleia Kim to the R state very often. This is why people think of the GPb form as less active than GPa. On the other hand, ATP and/or G6P are usually present at high enough concentration in cells that GPb is readily flipped into the T state (Figure 6.40).

GPa/GPb covalent regulation

The relative amounts of GPa and GPb largely govern the overall process of glycogen breakdown, since GPa tends to be active more often than GPb. It is i

Phosphorylase kinase itself has two covalent forms – phosphorylated (active) and dephosphorylated (inactive). It is phosphorylated by the enzyme Protein Kinase A (PKA -). Another way to activate the enzyme is allosterically with calcium (Figure 6.41). Phosphory- Figure 6.39 - Allosteric regulation of GPa Image by Aleia Kim Figure 6.40 - Allosteric regulation of GPb Image by Aleia Kim lase kinase is dephosphorylated by phosphoprotein phosphatase, the same enzyme that removes phosphate from GPa.

PKA and cAMPcAMP

PKA is activated by cAMP, which is, in turn, produced by adenylate cyclase after activation by a G-protein (See HERE for overview). G-proteins are activated ultimately by binding of ligands to specific membrane receptors called 7-TM receptors, also known as Gprotein coupled receptors. These are discussed in greater detail HERE. Common ligands for 7-TM receptors include epinephrine (binds β- adrenergic receptor) and glucagon (binds glucagon receptor). Epinephrine exerts its greatest effects on muscle and glucagon works preferentially on the liver. Thus, epinephrine and glucagon can activate glycogen breakdown by stimulating synthesis of cAMP followed by the cascade of events described above.

Turning off glycogen breakdown

Turning off signals is as important, if not more so, than turning them on. Glycogen is a precious resource. If its breakdown is not controlled, a lot of energy used in its synthesis is wasted. The steps in the glycogen breakdown regulatory pathway can be reversed at every level. First, the ligand (epinephrine or glucagon) can leave the receptor, turning off the stimulus. Second, the G-proteins have an inherent GTPase activity. GTP, of course, is what activates Gproteins, so a GTPase activity converts the GTP it is carrying to GDP and the G-protein becomes inactive. Thus, G-proteins turn off Figure 6.41 - Activation of phosphorylase kinase Image by Aleia Kim their own activity. Interfering with their ability to convert GTP to GDP can have dire consequences, including cancer in some cases.

Third, cells have phosphodiesterase enzymes (inhibited by caffeine) for breaking down cAMP. cAMP is needed to activate PKA, so breaking it down stops PKA from activating phosphorylase kinase. Fourth, the enzyme known as phosphoprotein phosphatase (also called PP1) plays a major role. It can remove phosphates from phosphorylase kinase (inactivating it) and form GPa, converting it to the less likely to be active GPb. Regulation of phosphoprotein phosphatase activity occurs at several levels. Two of these are shown in Figures 6.42 & 6.43.

In Figure 6.42, phosphoprotein phosphatase is shown being inactivated by phosphorylation of an inhibitor (called PI-1 - see below). This happens as a result of cascading actions from binding of epinephrine (or glucagon) to a cell's β-adrenergic receptor. Reversal of these actions occurs when insulin binds to the cell's insulin receptor, resulting in activation of phosphoprotein phosphatase.

PI-1

The inhibitor PI-1 can block activity of phosphpoprotein phosphatase only if it (PI-1) is phosphorylated. When PI-1 gets dephosphorylated, it no longer functions as an inhibitor, so phosphoprotein phosphatase be- Figure 6.42 - Inactivation of phosphoprotein phosphatase by protein kinase A via phosphorylation of PI-1 (Inhibitor) and the GM binding protein Image by Pehr

Jacobson Interactive Learning Module HERE comes active. Now, here is the clincher - PI-1 gets phosphorylated by PKA (thus, when epinephrine or glucagon binds to a cell) and gets dephosphorylated when insulin binds to a cell.

Another regulatory mechanism

Another way to regulate phosphoprotein phosphatase in the liver involves GPa directly (Figure 6.43). In liver cells, phosphoprotein phosphatase is bound to a protein called GL. GL can also bind to GPa. As shown in the figure, if the three proteins are complexed together (top of figure), then PP1 (phosphoprotein phosphatase) is inactive. When glucose is present (such as when the liver has made too much glucose), then the free glucose binds to the GPa and causes GPa to be released from the GL.

This has the effect of activating phosphoprotein phosphatase, which begins dephosphorylating enzymes. As shown in the figure, two such enzymes are GPa (making GPb) and glycogen synthase b, making glycogen synthase a. These dephosphorylations have opposite effects on the two enzymes, making GPb, which is less active and glycogen synthase a, which is much more active.

Glycogen synthesis

The anabolic pathway opposing glycogen breakdown is that of glycogen synthesis. Just Figure 6.43 - Regulation of phosphoprotein phosphatase (PP-1) activity by GPa Image by Penelope Irving as cells reciprocally regulate glycolysis and gluconeogenesis to prevent a futile cycle between these pathways, so too do cells use reciprocal schemes to regulate glycogen breakdown and synthesis.

Synthesis of glycogen starts with G1P, which is converted to an 'activated' intermediate, UDPglucose. This activated intermediate is what 'adds' the glucose to the growing glycogen chain in a reaction catalyzed by the enzyme known as glycogen synthase (Figure 6.44). Once the glucose is added to glycogen, the glycogen molecule may need to have branches inserted in it by the enzyme known as branching enzyme (Figure 6.45).

Steps

Let us first consider the steps in glycogen synthesis. 1) Glycogen synthesis from glucose involves phosphorylation to form G6P, and isomerization to form G1P (using phospho- igure 6.45 - Branch formation in glycogen by branching enzyme Image by Penelope Irving Figure 6.44 - Catalytic activity of glycogen synthase Image by Penelope Irving glucomutase, common to glycogen breakdown). G1P is reacted with UTP to form UDP-glucose in a reaction catalyzed by UDP-glucose pyrophosphorylase. Glycogen synthase catalyzes synthesis of glycogen by joining carbon #1 of the UDP-derived glucose onto the carbon #4 of the non-reducing end of a glycogen chain, to form the familiar α(1,4) glycogen links. Another product of the reaction is UDP.

“Primer” requirements

It is also worth noting, in passing, that glycogen synthase will only add glucose units from UDP-Glucose onto a preexisting glycogen chain that has at least four glucose residues. Linkage of the first few glucose units to form the minimal "primer" needed for glycogen synthase recognition is catalyzed by a protein called glycogenin, which attaches to the first glucose and catalyzes linkage of the first eight glucoses by α(1,4) bonds. 3) The characteristic α(1,6) branches of glycogen are the products of the enzyme known as branching enzyme. Branching enzyme breaks α(1,4) chains and carries the broken chain to the carbon #6 and forms an α(1,6) linkage (Figure 6.45).

Regulation of glycogen synthesis

The regulation of glycogen biosynthesis is reciprocal to that of glycogen breakdown. It also has a cascading covalent modification system similar to the glycogen breakdown system described above. In fact, part of the system is identical to glycogen breakdown. Epinephrine or glucagon signaling stimulates adenylate cyclase to make cAMP, which activates PKA. Figure 6.46 - Reciprocal regulation by the phosphorylation cascade - glycogen breakdown activated / glycogen synthesis inhibited Image by Penelope Irving

Effect of phosphorylation

In glycogen synthesis, protein kinase A phosphorylates the active form of glycogen synthase (GSa), and converts it into the usually inactive b form (called GSb).

Note the conventions for glycogen synthase and glycogen phosphorylase. For both enzymes, the more active forms are called the 'a' forms (GPa and GSa) and the less active forms are called the 'b' forms (GPb and GSb). The major difference, however, is that GPa has a phosphate, but GSa does not and GPb has no phosphate, but GSb does.

Thus phosphorylation and dephosphorylation have opposite effects on the enzymes of glycogen metabolism (Figure 6.46). This is the hallmark of reciprocal regulation. It is of note that the less active glycogen synthase form, GSb, can be activated by G6P. Recall that G6P had the exactly opposite effect on GPb.

Glycogen synthase, glycogen phosphorylase (and phosphorylase kinase) can all be dephosphorylated by the same enzyme - phosphoprotein phosphatase - and it is activated when insulin binds to its receptor in the cell membrane.

Big picture

In the big picture, binding of epinephrine or glucagon to appropriate cell receptors stimulates a phosphorylation cascade which simultaneously activates breakdown of glycogen by glycogen phosphorylase and inhibits synthesis of glycogen by glycogen synthase. Epinephrine, is also known as adrenalin, and the properties that adrenalin gives arise from a large temporary increase of blood glucose, which powers muscles.

On the other hand, insulin stimulates dephosphorylation by activating phosphoprotein phosphatase. Dephosphorylation reduces action of glycogen phosphorylase (less glycogen breakdown) and activates glycogen synthase (starts glycogen synthesis). Our bodies make glycogen when blood glucose levels rise. Since high blood glucose levels are harmful, insulin stimulates cells to take up glucose. In the liver and in muscle cells, the uptaken glucose is made into glycogen. Figure 6.47 - Cotton - the purest natural form of cellulose Wikipedia Interactive Learning Module HERE

Cellulose synthesis

Cellulose is synthesized as a result of catalysis by cellulose synthase. Like glycogen synthesis it requires an activated intermediate to add glucose residues and there are two possible ones - GDP-glucose and UDPglucose, depending on which cellulose synthase is involved. In plants, cellulose provides support to cell walls.

The reaction catalyzed is shown next where Cellulosen = a polymer of [(1→4)-β-Dglucosyl] n units long.

The GDP-glucose reaction is the same except with substitution of GDP-glucose for UDP-Figure 6.48 - The Pentose Phosphate Pathway - Enzymes - 1 = G6P dehydrogenase / 2 = 6-Phosphogluconolactonase / 3 = 6-PG dehydrogenase / 4a = Ribose 5-phosphate isomerase / 4b = Ribulose 5-phosphate 3-epimerase / 5,7 = Transketolase / 6 = Transaldolase UDP-glucose + Cellulosen UDP + Cellulosen+1 glucose. UDP-glucose for the reaction is obtained by catalysis of sucrose synthase. The enzyme is named for the reverse reaction.

Pentose phosphate pathway

The pentose phosphate pathway (PPP - also called the hexose monophosphate shunt) is an oxidative pathway involving sugars that is sometimes described as a parallel pathway to glycolysis. It is, in fact, a pathway with multiple inputs and outputs (Figure 6.48). PPP is also a major source of NADPH for biosynthetic reactions and can provide ribose-5-phosphate for nucleotide synthesis.

Though when drawn out, the pathway's "starting point" is often shown as glucose-6-phosphate (G6P), in fact there are multiple entry points including other glycolysis intermediates, such as fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (GLYAL-3-P), as well as less common sugar compounds with 4,5, and 7 carbons.

The multiple entry points and multiple outputs gives the cell tremendous flexibility to meet its needs by allowing it to use a variety of materials to make any of these products.

Oxidation #1

Beginning with G6P, PPP proceeds through its oxidative phase as follows:

The enzyme catalyzing the reaction is G6P dehydrogenase. It is the rate limiting step of the pathway and the enzyme is inhibited both by NADPH and acetyl-CoA. NADPH is important for anabolic pathways, such as fatty acid synthesis and also for maintaining glutathione in a reduced state. The latter is important in protection against damage from reactive oxygen species.

Deficiency of the G6P dehydrogenase enzyme is not rare, leading to acute hemolytic anemia, due to reduced NADPH concentration, and a reduced ability of the cell to disarm reactive oxygen species with glutathione. Reduced activity of the enzyme appears to have a protective effect against malarial infection, likely due to the increased fragility of the red blood cell membrane, which is then unable to sustain an infection by the parasite. Hydrolysis Reaction #2 is a hydrolysis and it is catalyzed by

Hydrolysis

Reaction #2 is a hydrolysis and it is catalyzed by 6-phosphogluconolactonase. The reac- Sucrose + UDP UDP-glucose + Fructose G6P + NADP+ 6-Phosphoglucono-δ-lactone + NADPH tion converts the circular 6-phosphoglucono- δ-lactone into the linear 6-phosphogluconate (6-PG) in preparation for oxidation in the next step.

Decarboxylation

Reaction #3 is the only decarboxylation in the PPP and the last oxidative step. It is catalyzed by 6-phosphogluconate dehydrogenase.

Mutations disabling the protein made from this gene negatively impact red blood cells. At this point, the oxidative phase of PPP is complete and the remaining reactions involve molecular rearrangements. Ru5P has two possible fates and these are each described below.

Isomerization

Reaction 4a: The enzyme catalyzing this reversible reaction is Ru5P isomerase (top of next column). It is important because this is the way cells make R-5-P for nucleotide synthesis. The R-5-P can also be used in other PPP reactions shown elsewhere.

Epimerization

Reaction 4b (catalyzed by Ru-5-P epimerase) is another source of a pentose sugars and provides an important substrate for subsequent reactions.

Transketolase reactions

The other reactions don't really have an order to them and whether they occur or not depends on cellular needs. The first enzyme, transketolase, is flexible in terms of its substrate/product combinations and is used not only in PPP, but also in the Calvin cycle of plants. It catalyzes the next two reactions

In the first reaction (above), two phosphorylated sugars of 5 carbons each are converted into one phosphorylated sugar of 3 carbons and one of 7 carbons. In the second (next page), a five carbon sugar phosphate and aRu-5-P Xylulose-5-phosphate (Xu-5-P) Xu-5-P + R-5-P GLYAL-3-P + Sedoheptulose-7-phosphate (S-7-P) 6-PG + NADP+ Ribulose-5-phosphate (Ru-5-P) + NADPH + CO2 6-Phosphoglucono-δ-lactone + H2O 6-phosphogluconate (6-PG) + H+ Ru-5-P Ribose-5-phosphate (R-5-P) four carbon sugar phosphate are rearranged into sugar phosphates with 3 and 6 carbons.

Glycolysis intermediates

In the reversible reactions of the pentose phosphate pathway, one can see how glycolysis intermediates can easily be rearranged and made into other sugars. Thus, GLYAL-3-P and F6P can be readily made into Ribose-5- phosphate for nucleotide synthesis.

Involvement of F6P in the pathway permits cells to continue making nucleotides (by making R-5-P) or tryptophan (by making E-4-P) even if the oxidative reactions of PPP are inhibited.

The last reaction is catalyzed by the enzyme known as transaldolase.

TPP co-factor

Transketolase uses thiamine pyrophosphate (TPP) to catalyze reactions. TPP's thiaFigure 6.49 - Intermediates of the pentose phosphate pathway Xu-5-P + Erythrose-4-phosphate (E-4-P) GLYAL-3-P + F6P GLYAL-3-P + S-7-P E-4-P + F6P zole ring's nitrogen and sulfur atoms on either side of a carbon, allow it to donate a proton and act as an acid, thus forming a carbanion, which gets stabilized by the adjacent tetravalent nitrogen (Figures 6.50 & 6.51)).

The stabilized carbanion plays important roles in the reaction mechanism of enzymes, such as transketolase that use TPP as a cofactor. Commonly, the carbanion acts as a nucleophile that attacks the carbonyl carbon of the substrate. Such is the case with transketolase. Attack by the carbanion breaks the carbonyl bond on the substrate and covalently links it to the ionized carbon of TPP, thus allowing it to "carry" the carbonyl group to the other substrate for attachment. In this way, two carbons are moved from Xu- 5-P to E-4-P to make F6P (from E-4-P) and GLYAL-3-P (from Xu-5-P). Similarly, S-7-P and GLYAL-3-P are made from R-5-P and Xu-5-P, respectively.

Thiamines

Thiamines are a class of compounds involved in catalysis of important respiration-related The Pentose Phosphate Pathway by Kevin Ahern I need erythrose phosphate And don't know what to do My cells are full of G-6-P And NADP too But I just hit upon a plan As simple as can be I'll run reactions through the path That's known as PPP In just two oxidations There's ribulose-5P Which morphs to other pentoses Each one attached to P The next step it is simple Deserving of some praise The pentose carbons mix and match Thanks to transketolase Glyceraldehyde's a product Sedoheptulose is too Each with a trailing phosphate But we are not quite through Now three plus seven is the same As adding six and four By swapping carbons back and forth There's erythrose-P and more At last I've got the thing I need From carbons trading places I'm happy that my cells are full Of some transaldolases Figure 6.50 - Thiamine pyrophosphate reactions in the citric acid cycle, pyruvate metabolism, the pentose phosphate pathway, and the Calvin cycle. Thiamine was the first water-soluble vitamin (B1) to be discovered via association with the peripheral nervous system disease known as Beriberi. Thiamine pyrophosphate (TPP) is an enzyme cofactor found in all living systems derived from thiamine by action of the enzyme thiamine diphosphokinase. TPP facilitates catalysis of several biochemical reactions essential for tissue respiration.

Deficiency of the vitamin is rare today, though people suffering from Crohn's disease, anorexia, alcoholism or undergoing kidney dialysis may develop deficiencies. TPP is required for the oxidative decarboxylation of pyruvate to form acetyl-CoA and similar reactions. Transketolase, an important enzyme in the pentose phosphate pathway, also uses it as a coenzyme. Besides these reactions, TPP is also required for oxidative decarboxylation of α-keto acids like α-ketoglutarate and branched-chain α-keto acids arising from metabolism of valine, isoleucine, and leucine. Figure 6.51 - Mechanism of action of thiamine pyrophosphate (TPP) - 1) Carbanion formation; 2) Nucleophilic attack; 3) Covalent attachment of carbonyl; 4) Transfer to second group; 5) Release of product and regeneration of TPP

TPP acts in the pyruvate dehydrogenase complex to assist in decarboxylation of pyruvate and "carrying" the activated acetaldehyde molecule to its attachment (and subsequent oxidation) to lipoamide. Central to TPP's function is the thiazolium ring, which stabilizes carbanion intermediates (through resonance) by acting as an electron sink (Figure 6.51). Such action facilitates breaking of carbon-carbon bonds such as occurs during decarboxylation of pyruvate to produce the activated acetaldehyde.

Thiamine deficiency

Thiamine is integral to respiration and is needed in every cell. Acute deficiency of thiamine leads to numerous problems - the best known condition is beriberi, whose symptoms include weight loss, weakness, swelling, neurological issues, and irregular heart rhythms. Figure 6.52 - The Calvin cycle - The resynthesis phase has multiple steps and is described below. Image by Aleia Kim

Causes of deficiency include poor nutrition, significant intake of foods containing the enzyme known as thiaminase, foods with compounds that counter thiamine action (tea, coffee), and chronic diseases, including diabetes, gastrointestinal diseases, persistent vomiting. People with severe alcoholism often are deficient in thiamine.

Calvin cycle

The Calvin cycle (Figure 6.52) is a metabolic pathway occurring exclusively in photosynthetic organisms. Commonly referred to as the "Dark Cycle" or the Light-Independent Cycle, the Calvin cycle does not actually occur in the dark. The cell/chloroplast simply is not directly using light energy to drive it.

Assimilation

It is in the Calvin cycle of photosynthesis that carbon dioxide is taken from the atmosphere and ultimately built into glucose (or other sugars). Reactions of the Calvin cycle take place in regions of the chloroplast known as the stroma, the fluid areas outside of the thylakoid membranes. The cycle can be broken into three phases

1) assimilation of CO2

2) reduction reactions

3) regeneration of the starting material, ribulose 1,5 bisphosphate (Ru1,5BP).

Though reduction of carbon dioxide to glucose ultimately requires electrons from twelve molecules of NADPH (and 18 ATPs), it is confusing because one reduction occurs 12 times (1,3 BPG to GLYAL-3P) to input the overall reduction necessary to make one glucose.

Carbon dioxide

Another reason students find the pathway confusing is because the carbon dioxide molecules are absorbed one at a time into six different molecules of Ru1,5BP. At no point are the six carbons ever together in the same molecule to make a single glucose.

Instead, six molecules of Ru1,5BP (30 carbons) gain six more carbons via carbon dioxide and then split into 12 molecules of 3-phosphoglycerate (36 carbons). The gain of six carbons allows two three carbon molecules to be produced in excess for each turn of the cycle. These two molecules molecules are then converted into glucose using the enzymes of gluconeogenesis. The other ten molecules of 3-PG are used to regenerate the six molecules of Ru1,5BP. Figure 6.53 - Rubisco, the most abundant enzyme on Earth

Cyclic pathway

Like the citric acid cycle, the Calvin cycle doesn't really have a starting or ending point, but can we think of the first reaction as the fixation of carbon dioxide to Ru1,5BP. This reaction is catalyzed by the enzyme known as ribulose-1,5 bisphosphate carboxylase (RUBISCO - Figure 6.53). The resulting six carbon intermediate is unstable and is rapidly converted to two molecules of 3-phosphoglycerate.

As noted, if one starts with 6 molecules of Ru1,5BP and makes 12 molecules of 3-PG, the extra 6 carbons that are a part of the cycle can be shunted off as two three-carbon molecules of glyceraldehyde-3-phosphate (GLYAL3P) to gluconeogenesis, leaving behind 10 molecules to be reconverted into 6 moleFigure 6.54 - Resynthesis phase of the Calvin cycle - All paths lead to regenerating Ru1,5BP, which is the aim of the resynthesis phase. Glycolysis/gluconeogenesis intermediates shown in blue. Enzyme numbers explained in text. cules of Ru1,5BP. This occurs in what is called the resynthesis phase.

Resynthesis phase

The resynthesis phase (Figure 6.54) requires multiple steps, but only utilizes two enzymes unique to plants - sedoheptulose-1,7 bisphosphatase and phosphoribulokinase. RUBISCO is the third (and only other) enzyme of the pathway that is unique to plants.

All of the other enzymes of the pathway are common to plants and animals and include some found in the pentose phosphate pathway and gluconeogenesis. Enzymes shown as numbers in Figure 6.54 are as follows (enzymes unique to plants in green):

1 - Phosphoglycerate kinase

2 - G3PDH

3 - Triosephosphate Isomerase

4 - Aldolase

5 - Fructose 1,6 bisphosphatase

6 - Transketolase

7 - Phosphopentose Epimerase

8 - Phosphoribulokinase

9 - Sedoheptulose 1,7 bisphosphatase

10 - Phosphopentose Isomerase

Reactions

The resynthesis phase begins with conversion of the 3-PG molecules into GLYAL3P (there are actually 10 GLYAL3P molecules involved in resynthesis, as noted above, but we are omitting numbers to try to help students to see the bigger picture. Suffice it to say that there are sufficient quantities of all of the molecules to complete the reactions described). Some GLYAL3P is converted to DHAP by triose phosphate isomerase. Some DHAPs are converted (via gluconeogenesis) to F6P (one phosphate is lost for each F6P).

Two carbons from F6P are given to GLYAL3P to create E-4P and Xu-5P (reversal of PPP reaction). E- 4P combines with DHAP to form sedoheptulose-1,7 bisphosphate (S1,7BP). The phosphate at position #1 is Figure 6.55 - Use of CO2 (Calvin cycle) vs. O2 (photorespiration) by RUBISCO. Image by Pehr Jacobson cleaved by sedoheptulose-1,7 bisphosphatase to yield S-7-P. Transketolase (another PPP enzyme) catalyzes transfer to two carbons from S-7-P to GLYAL3P to yield Xu-5P and R5P.

Phosphopentose isomerase catalyzes conversion of R5P to Ru5P and phosphopentose epimerase similarly converts Xu-5P to Ru5P. Finally, phosphoribulokinase transfers a phosphate to Ru5P (from ATP) to yield Ru1,5BP.

Photorespiration

In the Calvin cycle of photosynthesis, the enzyme ribulose-1,5-bisphosphate carboxylase (RUBISCO) catalyzes the addition of carbon dioxide to ribulose-1,5- bisphosphate (Ru1,5BP) to create two molecules of 3-phosphoglycerate. Molecular oxygen (O2), however, competes with CO2 for this enzyme, so about 25% of the time, the molecule that gets added is not CO2, but rather O2 (Figure 6.55). When this happens, the following reaction occurs

This is the first step in the process known as photorespiration. The process of photorespiration is inefficient relative to the carboxylation of Ru1,5BP. Phosphoglycolate is converted to glyoxylate in the glyoxysome and then transamination of that yields glycine. Two glycines can combine in a complicated coupled set of reactions in the mitochondrion shown next. Figure 6.56 - Maize - a C4 plant Ru1,5BP + O2 Phosphoglycolate + 3-phosphoglycerate + 2H+ 2 Glycine + NAD+ + H2O Serine + CO2 + NH3 + NADH + H+

Deamination and reduction of serine yields pyruvate, which can be then be converted back to 3-phosphoglycerate. The end point of oxygenation of Ru1,5BP is the same as the carboxylation of Ru1,5BP reactions, but there are significant energy costs associated with it, making the process less efficient.

C4 plants

The Calvin cycle is the means by which plants assimilate carbon dioxide from the atmosphere, ultimately into glucose. Plants use two general strategies for doing so. The first is employed by plants called C3 plants (most plants) and it simply involves the pathway described above. They are called C3 plants because the first stable intermediate after absorbing carbon dioxide contains three carbons - 3-phosphoglycerate. Another class of plants, called C4 plants (Figure 6.56) employ a novel strategy for concentrating the Figure 6.57 - Assimilation of CO2 by C4 plants Image by Aleia Kim CO2 prior to assimilation. C4 plants are generally found in hot, dry environments where conditions would otherwise favor the wasteful photorespiration reactions of RUBISCO and loss of water.

Capture by PEP

In C4 plants, carbon dioxide is captured in special mesophyll cells first by phosphoenolpyruvate (PEP) to make oxaloacetate (contains four carbons and gives the C4 plants their name - Figure 6.57). The oxaloacetate is converted to malate and transported into bundle sheath cells where the carbon dioxide is released and captured by Ru1,5BP, as in C3 plants. The Calvin cycle proceeds from there. The advantage of the C4 plant scheme is that it allows concentration of carbon dioxide while minimizing loss of water and photorespiration.

Peptidoglycan synthesis

Bacterial cell walls contain a layer of protection known as the peptidoglycan layer. Assembly of the layer begins in the cytoplasm.

Steps in the process follow

1. Donation of an amine from glutamine to fructose-6- phosphate and isomerization to make glucosamine-6- phosphate.

2. Donation of an acetyl group from acetyl-CoA to make N-acetylglucosamine-6- phosphate

3. Isomerization of N-acetylglucosamine-6- phosphate makes N-acetylglucosamine-1- phosphate Figure 6.58 - Peptidoglycan layer in a bacterial outer cell wall Wikipedia

4. UTP combines with N-acetylglucosamine-1-phosphate to make UDP-N-acetyl-glucosamine-1- phosphate

5. Addition of PEP and electrons from NADPH yields UDP-Nacetylmuramic acid

6. A pentapeptide or tetrapeptide chain is attached to the UDP-Nacetylmuramic acid. The sequence varies a bit between species, but commonly is L-Ala - D-Glu - L-Lys - DAla - D-Ala

7. Dolichol phosphate replaces UMP on the UDP-N-acetylmuramic acid-pentapeptide.

8. UDP-N-acetyl-glucosamine donates a glucose to the Nacetylmuramic acid part of the Dolichol-PP-N-acetylmuramic acidpentapeptide

9. A pentapeptide chain of glycines (pentaglycine) is linked to lysine of the pentapeptide chain to create a Dolichol-PP-Nacetylmuramic acid-N-acetylglucosaminedecapeptide. The pentaglycine serves as cross links in the overall structure.

10. Dolichol-PP is removed to yield Nacetylmuramic acid-N-acetylglucosaminedecapeptide Figure 6.60 - Catalytic activity of DDtranspeptidase Wikipedia Figure 6.59 - Penicillin

11. This last group is added to the growing peptidoglycan network by joining the pentaglycine of one chain to the tetrapeptide/ pentapeptide of another.

The enzyme catalyzing the addition of the N-acetylmuramic acid-N-acetylglucosaminedecapeptide to the network in the last step is DD-transpeptidase. This is the cellular enzyme targeted by penicillin and its derivatives. One reason penicillin is so effective is because synthesis of a peptidoglycan cell wall for a single bacterium requires millions of cycles of reactions above. Even slowing down the process can have a major effect on bacterial growth. On the flip side, resistance to penicillin and derivatives arises as a result of mutations in one enzyme - the transpeptidase.

Metabolons

At this point, it is appropriate to bring up the concept of metabolons. Metabolons are cellular complexes containing multiple enzymes of a metabolic pathway that appear to be arranged so that the product of one enzymatic reaction is passed directly as substrate to the enzyme that catalyzes the next reaction in the metabolic pathway. The structural complexes are temporary and are held together by non-covalent forces.

Metabolons appear to offer advantages of reducing the amount of water needed to hydrate enzymes. Activity of enzymes in the complex is increased. Most of the basic metabolic pathways are thought to use metabolons. They include glycolysis, the citric acid cycle, nucleotide metabolism, glycogen synthesis, steroid synthesis, DNA synthesis, RNA synthesis, the urea cycle, and the process of electron transport.

Hypoxia

Hypoxia occurs when the body or a region of it has an insufficient oxygen supply. Varia- Figure 6.61 - Hypoxia inducible factor tions in arterial oxygen concentrations in normal physiology may lead to hypoxia, for example, during hypoventilation training or strenuous physical exercise. Generalized hypoxia may appear in healthy people when at high altitudes. Cancer cells, which may be undergoing faster respiration than surrounding tissues may also tend to be hypoxic. Hypoxia is an important consideration for sugar metabolism due to the ability of cells to change their sugar metabolism (fermentation) when these conditions exist.

The body's response to hypoxia is to produce Hypoxia-Inducible Factors (HIFs), which are transcription factors that induce expression of genes to help cells adapt to the hypoxic conditions. Many of the genes activated by HIFs are enzymes of glycolysis and GLUTs (glucose transport proteins). The combination of these gene products allows cells to 1) import more glucose and 2) metabolize it more rapidly when it arrives. This is to be expected because anaerobic sugar metabolism is only about 1/15th as efficient as aerobic metabolism. Consequently, it requires much more sugar metabolism to keep the cancer cells alive. A recently discovered protein called cytoglobin is believed to help assist in hypoxia by facilitating transfer of oxygen from arteries to the brain.

Covalent modification

HIFs are regulated partly by an interesting covalent modification. When oxygen concentration is high, the enzyme prolyl hydroxylase will hydroxylate proline residues in HIFs. This stimulates the protein degradation system (proteasome) to degrade them. When oxygen concentration is low, the hydroxylation occurs to a much lower extent (or does not occur at all), reducing/stopping degradation of HIFs and allowing them to activate genes. In this way, the concentration of HIFs is kept high under low oxygen concentration (to activate HIF genes) and low under high oxygen concentrations (to stop synthesis of HIF genes).

This page titled 6.2: Metabolism - Sugars is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **6.1: Metabolism - Sugars** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

6.3: Citric Acid Cycle and Related Pathways

Source: BiochemFFA_6_2.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

Citric acid cycle

The primary catabolic pathway in the body is the citric acid cycle because it is here that oxidation to carbon dioxide occurs for breakdown products of the cell's major building blocks - sugars, fatty acids, and amino acids. The pathway is cyclic (Figure 6.63) and thus, doesn't really have a starting or ending point. All of the reactions occur in mitochondria, though one enzyme is embedded in the organelle's inner membrane. As needs change, cells may use a subset of the reactions of the cycle to produce the desired molecules via partial cycle rather than to run the entire cycle.

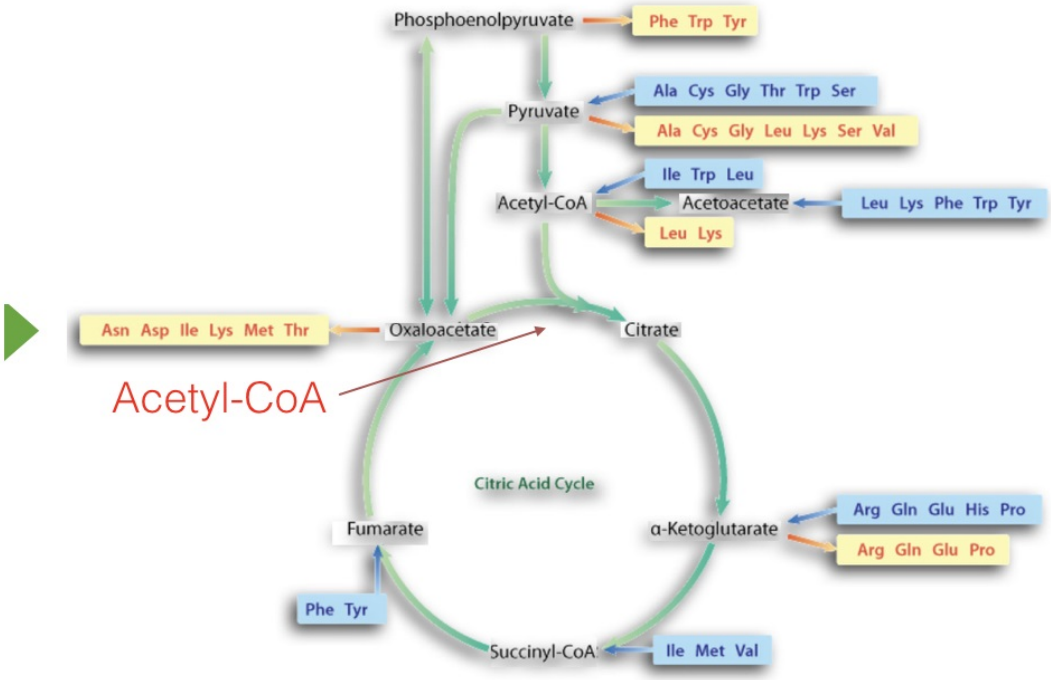


Figure 6.63 - Amino acid metabolism and the citric acid cycle. Amino acids boxed in yellow are made from the indicated intermediate. Amino acids in blue are made into the intermediate in catabolism. Image by Aleia Kim

Acetyl-CoA

The molecule "feeding" the citric acid cycle is acetyl-CoA and it can be obtained from pyruvate (from glycolysis), from fatty acid β-oxidation, from ketone bodies, and from amino acid metabolism. Molecules from other pathways feeding into the citric acid cycle for catabolism make the citric acid cycle 'cataplerotic'. It is worth noting that acetyl-CoA has very different fates, depending on the cell's energy status/needs (see HERE). The description below describes oxidation (catabolism) in citric acid cycle.

Anabolically, acetyl-CoA is also very important for providing building blocks for synthesis of fatty acids, ketone bodies, amino acids and cholesterol. Other citric acid cycle intermediates are also important in amino acid metabolism (Figure 6.63), heme synthesis, electron shuttling, and shuttling of acetyl-CoA across the mitochondrial inner membrane. The ability of the citric acid cycle to supply intermediates to pathways gives rise to the term 'anaplerotic.' It means 'to fill up.' Before discussing the citric acid cycle, it is important to first describe one important enzyme complex that is a major source of acetyl-CoA for the cycle.

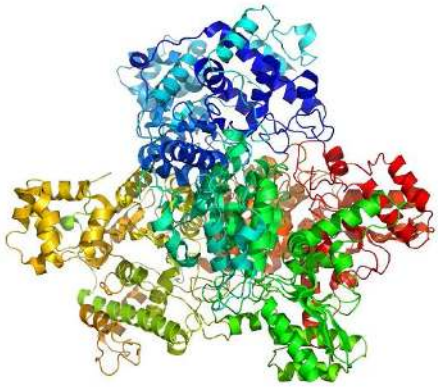


Figure 6.64 - E1 Subunit of Pyruvate Dehydrogenase. Wikipedia

Pyruvate decarboxylation

The pyruvate dehydrogenase enzyme is a complex of multiple copies of three subunits that catalyze the decarboxylation of pyruvate to form acetyl-CoA. The reaction mechanism requires use of five coenzymes. Pyruvate dehydrogenase is an enormous complex in mammals with a size five times greater than ribosomes.

Subunits

The three subunits are designated by E1, E2, and E3. E2 is also referred to as dihydrolipoamide acetyltransferase and E3 is more precisely called dihydrolipoyl dehydrogenase. Confusion arises with the name for E1. Some call it pyruvate dehydrogenase and others give it the name pyruvate decarboxylase. We will use pyruvate decarboxylase solely to refer to E1 and pyruvate dehydrogenase only to refer to the complex of E1, E2, and E3.

The catalytic actions of pyruvate dehydrogenase can be broken down into three steps, each taking place on one of the subunits. The steps, sequentially occurring on E1, E2, and E3, are 1) decarboxylation of pyruvate; 2) oxidation of the decarboxylated product; and 3) transfer of electrons to ultimately form NADH (Figure 6.65).

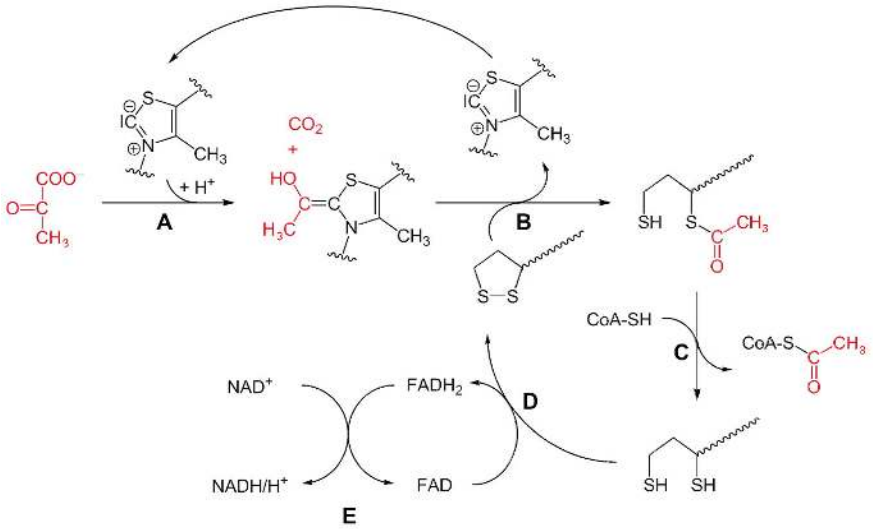


Figure 6.65 - Mechanism of action of pyruvate decarboxylation and oxidation by pyruvate dehydrogenase.

Catalysis

The catalytic process begins after binding of the pyruvate substrate with activation of the thiamine pyrophosphate coenzyme through formation of an ylide intermediate. The nucleophilic carbanion of the ylide attacks the electrophilic ketone carbon on the

pyruvate, releasing carbon dioxide and creating an enol that loses a proton on the carbon to become a 1,3 dipole that includes the positively charged nitrogen of the thiamine. The reaction (step A in Figure 6.65) is a non-oxidative decarboxylation. Oxidation of the two carbon hydroxyethyl unit occurs in the transfer to the lipoamide.

Reductive acetylation

Reductive acetylation occurs next (Step B) as the 2-carbon hydroxyethyl unit is transferred to lipoamide on E2. (Lipoamide is the name for a molecule of lipoic acid covalently attached to a lysine side chain in the E2 subunit). In prokaryotes in the absence of oxygen, the hydroxyethyl group is not passed to lipoamide, but instead is released as free acetaldehyde , which can accept electrons from NADH (catalyzed by alcohol dehydrogenase) and become ethanol in the process of fermentation. In the presence of oxygen in almost all aerobic organisms, the process continues with transfer of the hydroxyethyl unit to E2 and continuation of the cycle below.

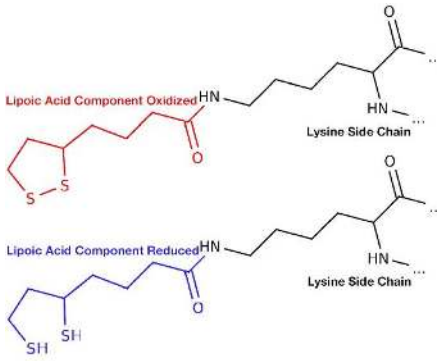


Figure 6.66 - Oxidized and reduced structures of lipoamide (lipoic acid linked to lysine)

Oxidation step

Transfer of the hydroxyethyl group from E1 to the lipoamide coenzyme in E2 is an oxidation, with transfer of electrons from the hydroxyethyl group to lipoamide's disulfide (reducing it) and formation on the lipoamide of an acetyl-thioester (oxidizing it).

The acetyl group is then transferred from lipoamide to coenzyme A in E2 (Step C in Figure 6.65), forming acetyl-CoA, which is released and leaving reduced sulfhydryls on the lipoamide. In order for the enzyme to return to its original state, the disulfide bond on lipoamide must be re-formed. This occurs with transfer of electrons from reduced lipoamide to an FAD covalently bound to E3 (Step D). This reduces FAD to FADH2.

Formation of NADH

In the last step in the process, electrons from FADH2 are transferred to external NAD+, forming NADH (Step E) and completing the overall cycle. Then enzyme can then begin another catalytic round by binding to a pyruvate.

Pyruvate dehydrogenase regulation

Pyruvate deyhdrogenase is regulated both allosterically and by covalent modification - phosphorylation / dephosphorylation. Regulation of pyruvate dehydrogenase, whether by allosteric or covalent mechanisms has the same strategy. Indicators of high energy shut down the enzyme, whereas indicators of low energy stimulate it. For allosteric regulation, the high energy indicators affecting the enzyme are ATP, acetyl-CoA, NADH, and fatty acids, which inhibit it. AMP, Coenzyme A, NAD+, and calcium, on the other hand, stimulate it (Figure 6.67).

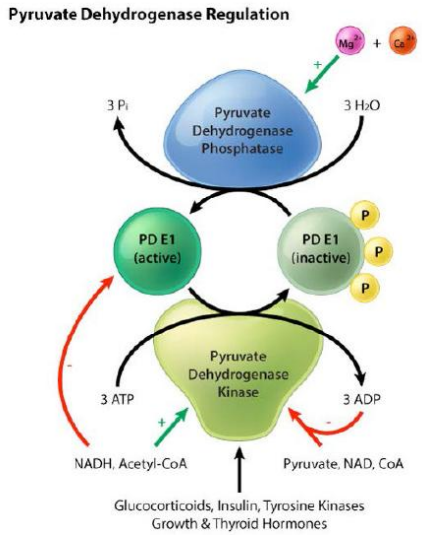


Figure 6.67 - Regulation scheme for pyruvate dehydrogenase (PD). Image by Aleia Kim

Covalent modification

Covalent modification regulation of pyruvate dehydrogenase is a bit more complicated. It occurs as a result of phosphorylation by pyruvate dehydrogenase kinase (PDK - Figure 6.67) or dephosphorylation by pyruvate dehydrogenase phosphatase (PDP).

PDK puts phosphate on any one of three serine residues on the E1 subunit, which causes pyruvate kinase to not be able to perform its first step of catalysis - the decarboxylation of pyruvate. PDP can remove those phosphates. PDK is allosterically activated in the mitochondrial matrix when NADH and acetyl-CoA concentrations rise.

Product inhibition

Thus, the products of the pyruvate dehydrogenase reaction inhibit the production of more products by favoring its phosphorylation by PDK. Pyruvate, a substrate of pyruvate dehydrogenase, inhibits PDK, so increasing concentrations of substrate activate pyruvate dehydrogenase by reducing its phosphorylation by PDK. As concentrations of NADH and acetyl-CoA fall, PDP associates with pyruvate kinase and removes the phosphate on the serine on the E1 subunit.

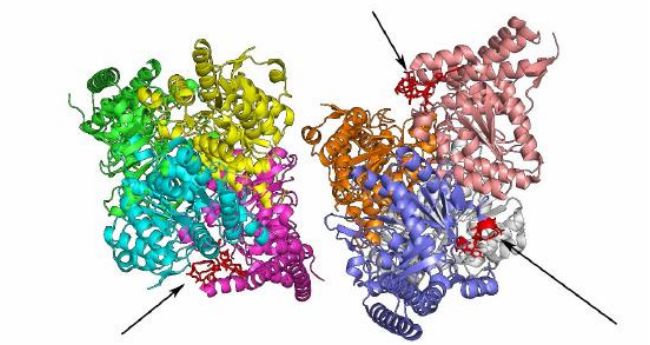


Figure 6.68 - Pyruvate dehydrogenase complex with three phosphorylation sites in red marked by arrows.Wikipedia

Low concentrations of NADH and acetyl-CoA are necessary for PDP to remain on the enzyme. When those concentrations rise, PDP dissociates and PDK gains access to the serine for phosphorylation. Insulin and calcium can also activate the PDP. This is very important in muscle tissue, since calcium is a signal for muscular contraction, which requires energy. Insulin also also activates pyruvate kinase and the glycolysis pathway to use internalized glucose. It should be noted that the cAMP phosphorylation cascade from the β-adrenergic receptor has no effect on pyruvate kinase, though the insulin cascade does, in fact, affect PDP and pyruvate kinase.

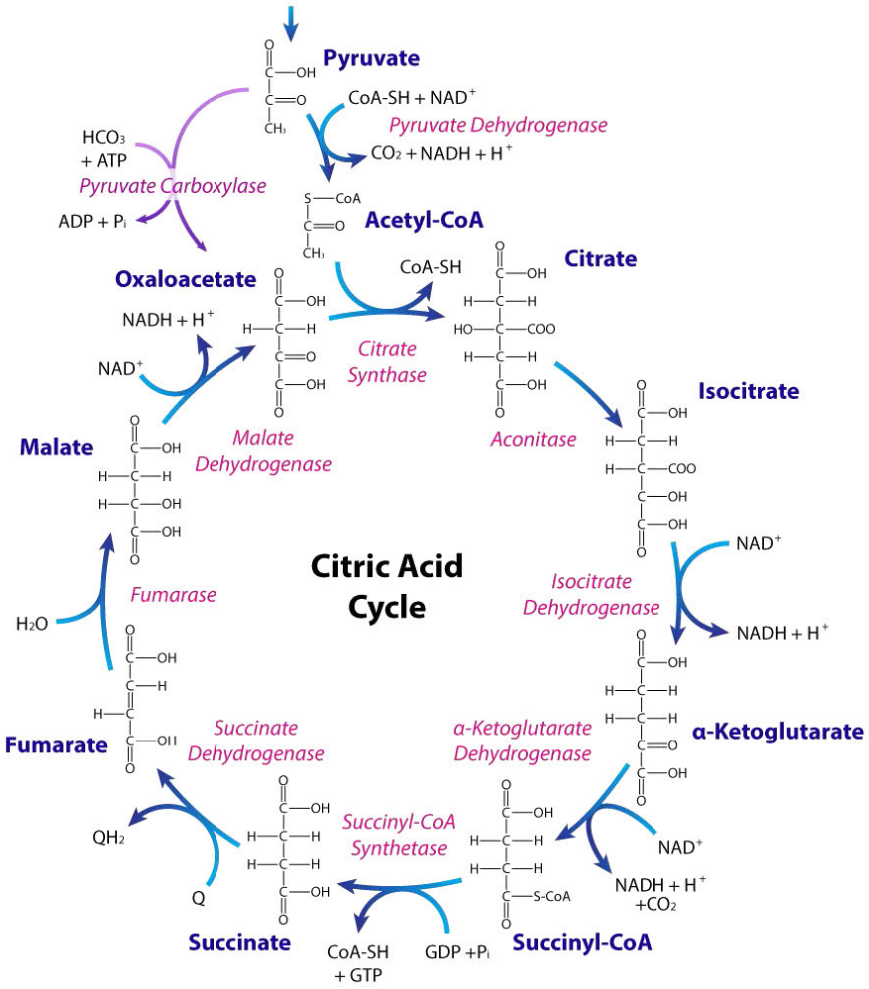


Figure 6.69 - The citric acid cycle. Image by Aleia Kim

Citric acid cycle reactions

Focusing on the pathway itself (Figure 6.69), the usual point to start discussion is addition of acetyl-CoA to oxaloacetate (OAA) to form citrate.

Acetyl-CoA for the pathway can come from a variety of sources. The reaction joining it to OAA is catalyzed by citrate synthase and the ΔG°' is fairly negative. This, in turn, helps to "pull" the malate dehydrogenase reaction preceding it in the cycle.

In the next reaction, citrate is isomerized to isocitrate by action of the enzyme called aconitase.

Isocitrate is a branch point in plants and bacteria for the glyoxylate cycle (see HERE). Oxidative decarboxylation of isocitrate by isocitrate dehydrogenase produces the first NADH and yields α-ketoglutarate.

This five carbon intermediate is a branch point for synthesis of the amino acid glutamate. In addition, glutamate can also be made easily into this intermediate in the reverse reaction. Decarboxylation of α-ketoglutarate produces succinyl-CoA and is catalyzed by α-ketoglutarate dehydrogenase.

The enzyme α-ketoglutarate dehydrogenase is structurally very similar to pyruvate dehydrogenase and employs the same five coenzymes – NAD+, FAD, CoA-SH, thiamine pyrophosphate, and lipoamide.

Regeneration of oxaloacetate

The remainder of the citric acid cycle involves conversion of the four carbon succinyl-CoA into oxaloacetate. Succinyl-CoA is a branch point for the synthesis of heme (see HERE). Succinyl-CoA is converted to succinate in a reaction catalyzed by succinyl-

CoA synthetase (named for the reverse reaction) and a GTP is produced, as well – the only substrate level phosphorylation in the cycle.

The energy for the synthesis of the GTP comes from hydrolysis of the high energy thioester bond between succinate and the CoA-SH. Evidence for the high energy of a thioester bond is also evident in the citrate synthase reaction, which is also very energetically favorable. Succinate is also produced by metabolism of odd-chain fatty acids (see HERE).

Succinate Oxidation

Oxidation of succinate occurs in the next step, catalyzed by succinate dehydrogenase. This interesting enzyme both catalyzes this reaction and participates in the electron transport system, funneling electrons from the FADH2 it gains in the reaction to coenzyme Q. The product of the reaction, fumarate, gains a water across its trans double bond in the next reaction, catalyzed by fumarase to form malate.

Fumarate is also a byproduct of nucleotide metabolism and of the urea cycle. Malate is important also for transporting electrons across membranes in the malate-aspartate shuttle (see HERE) and in ferrying carbon dioxide from mesophyll cells to bundle sheath cells in C4 plants (see HERE).

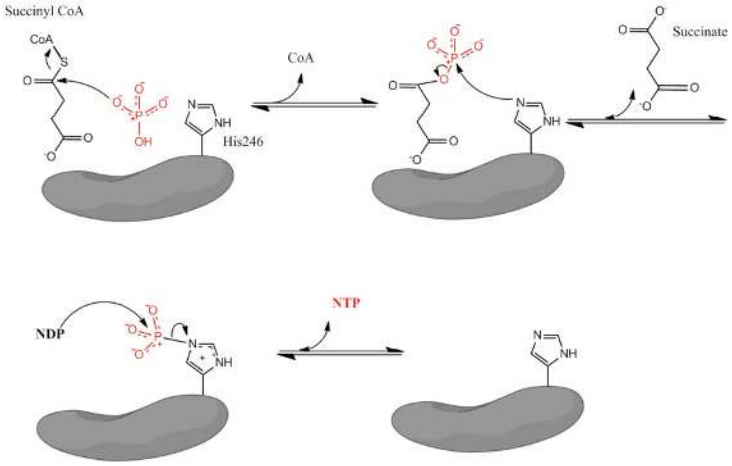


Figure 6.70 - Succinyl-CoA synthetase mechanism

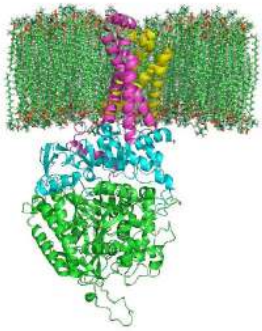


Figure 6.71 - Succinate dehydrogenase embedded in the mitochondrial inner membrane (top). Wikipedia

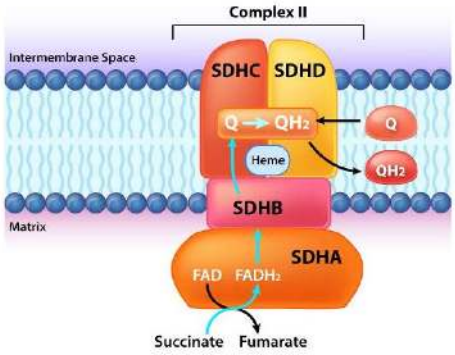


Figure 6.72 - Succinate dehydrogenase reaction. Image by Aleia Kim

Rare oxidation

Conversion of malate to oxaloacetate by malate dehydrogenase is a rare biological oxidation that has a ΔG°' with a positive value (29.7 kJ/mol).

The reaction is 'pulled' by the energetically favorable conversion of oxaloacetate to citrate in the citrate synthase reaction described above. Oxaloacetate intersects other important pathways, including amino acid metabolism (readily converted to aspartic acid), transamination (nitrogen movement) and gluconeogenesis.

It is worth noting that reversal of the citric acid cycle theoretically provides a mechanism for assimilating CO2. In fact, this reversal has been noted in both anaerobic and microaerobic bacteria, where it is called the Arnon-Buchanan cycle (Figure 6.73).

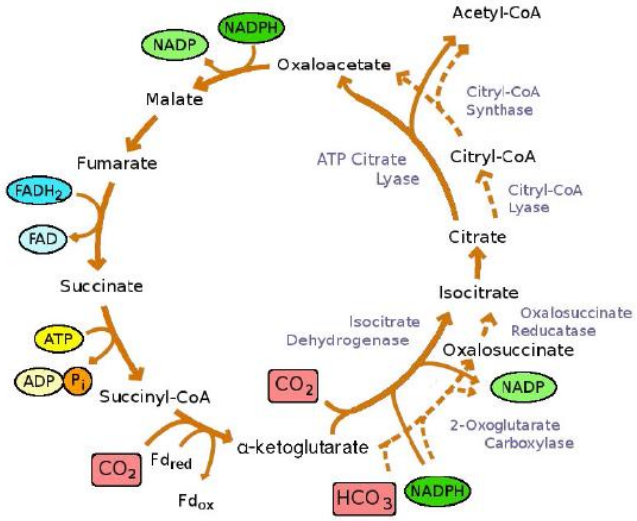


Figure 6.73 - Arnon-Buchanon cycle. Alternative enzymes shown on right in lavender. Fd = ferredoxin. Wikipedia

Regulation of the citric acid cycle

Allosteric regulation of the citric acid cycle is pretty straightforward. The molecules involved are all substrates/products of the pathway or molecules involved in energy transfer. Substrates/products that regulate or affect the pathway include acetyl-CoA and succinyl-CoA .

Inhibitors and activators

High energy molecular indicators, such as ATP and NADH will tend to inhibit the cycle and low energy indicators (NAD+, AMP, and ADP) will tend to activate the cycle. Pyruvate dehydrogenase, which catalyzes formation of acetyl-CoA for entry into the cycle is allosterically inhibited by its product (acetyl-CoA), as well as by NADH and ATP.

Regulated enzymes

Regulated enzymes in the cycle include citrate synthase (inhibited by NADH, ATP, and succinyl-CoA), isocitrate dehydrogenase (inhibited by ATP, activated by ADP and NAD+), and α-ketoglutarate dehydrogenase (inhibited by NADH and succinyl-CoA and activated by AMP).

Anaplerotic/cataplerotic pathway

The citric acid cycle is an important catabolic pathway oxidizing acetyl-CoA into CO2 and generating ATP, but it is also an important source of molecules needed by cells and a mechanism for extracting energy from amino acids in protein breakdown and other breakdown products. This ability of the citric acid cycle to supply molecules as needed and to absorb metabolic byproducts gives great flexibility to cells. When citric acid cycle intermediates are taken from the pathway to make other molecules, the term used to describe this is cataplerotic, whereas when molecules are added to the pathway, the process is described as anaplerotic.

Cataplerotic molecules

The citric acid cycle's primary cataplerotic molecules include α-ketoglutarate, succinyl-CoA, and oxaloacetate. Transamination of α-ketoglutarate and oxaloacetate produces the amino acids glutamate and aspartic acid, respectively. Oxaloacetate is important for the production of glucose in gluconeogenesis.

Glutamate plays a very important role in the movement of nitrogen through cells via glutamine and other molecules and is also needed for purine synthesis. Aspartate is a precursor of other amino acids and for production of pyrimidine nucleotides. Succinyl-CoA is necessary for the synthesis of porphyrins, such as the heme groups in hemoglobin, myoglobin and cytochromes.

Citrate is an important source of acetyl-CoA for making fatty acids. When the citrate concentration is high (as when the citric acid cycle is moving slowly or is stopped), it gets shuttled across the mitochondrial membrane into the cytoplasm and broken down by the enzyme citrate lyase to oxaloacetate and acetyl-CoA. The latter is a precursor for fatty acid synthesis in the cytoplasm.

Anaplerotic molecules

Anaplerotic molecules replenishing citric acid cycle intermediates include acetyl-CoA (made in many pathways, including fatty acid oxidation, pyruvate decarboxylation, amino acid catabolism, and breakdown of ketone bodies), α-ketoglutarate (from amino acid metabolism), succinyl-CoA (from propionic acid metabolism), fumarate (from the urea cycle and purine metabolism), malate (carboxylation of PEP in plants), and oxaloacetate (many sources, including amino acid catabolism and pyruvate carboxylase action on pyruvate in gluconeogenesis)

Glyoxylate cycle

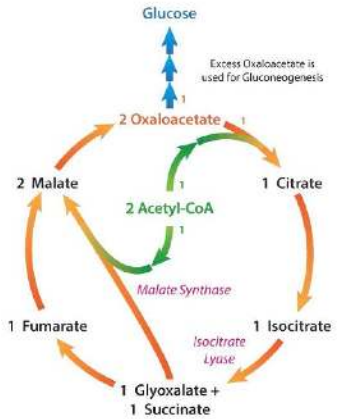


Figure 6.74 - Overview of the glyoxylate cycle. Image by Aleia Kim

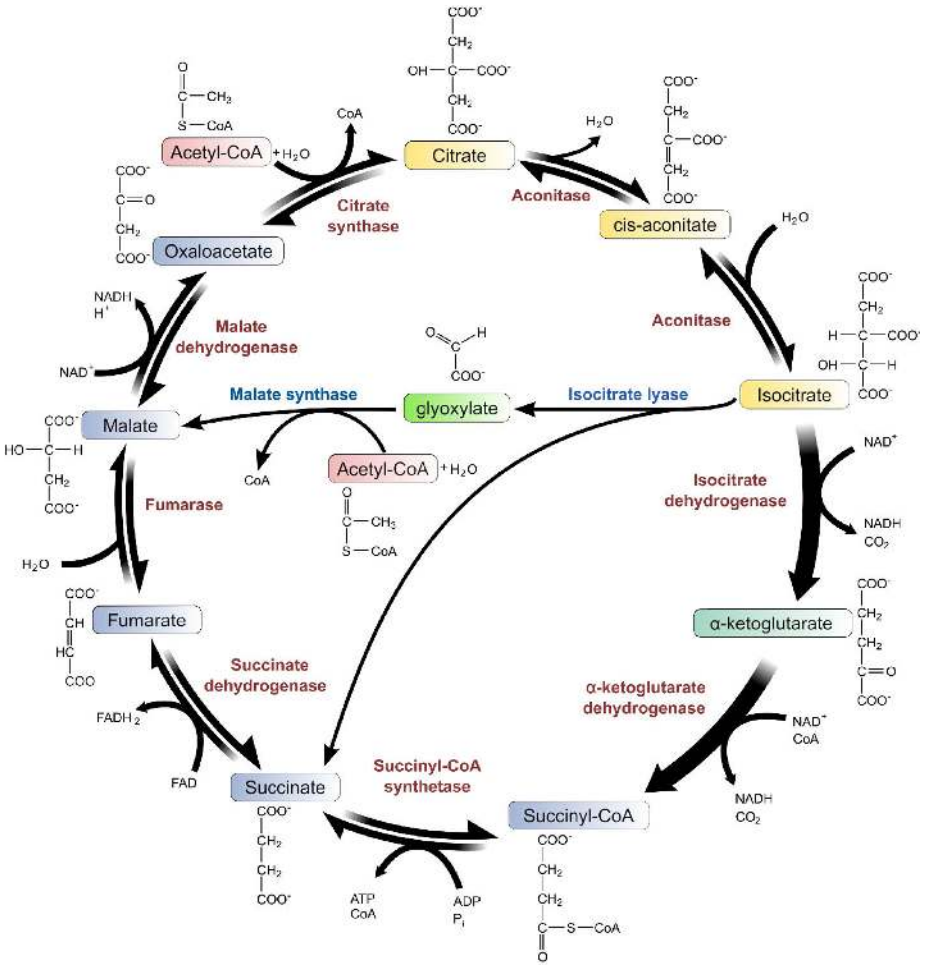


Figure 6.75 - Reactions of the glyoxylate cycle. Wikipedia

A pathway related to the citric acid cycle found only in plants and bacteria is the glyoxylate cycle (Figures 6.74 & 6.75). The glyoxylate cycle, which bypasses the decarboxylation reactions while using most of the non-decarboxylation reactions of the citric acid cycle, does not operate in animals, because they lack two enzymes necessary for it – isocitrate lyase and malate synthase. The cycle occurs in specialized plant peroxisomes called glyoxysomes. Isocitrate lyase catalyzes the conversion of isocitrate into succinate and glyoxylate. Because of this, all six carbons of the citric acid cycle survive each turn of the cycle and do not end up as carbon dioxide.

Succinate continues through the remaining reactions to produce oxaloacetate. Glyoxylate combines with another acetyl-CoA (one acetyl-CoA was used to start the cycle) to create malate (catalyzed by malate synthase). Malate can, in turn, be oxidized to oxaloacetate.

It is at this point that the glyoxylate pathway's contrast with the citric acid cycle is apparent. After one turn of the citric acid cycle, a single oxaloacetate is produced and it balances the single one used in the first reaction of the cycle. Thus, in the citric acid cycle, there is no net production of oxaloacetate in each turn of the cycle.

Net oxaloacetate production

On the other hand, thanks to assimilation of carbons from two acetyl-CoA molecules, each turn of the glyoxylate cycle results in two oxaloacetates being produced, after starting with one. The extra oxaloacetate of the glyoxylate cycle can be used to make other molecules, including glucose in gluconeogenesis. This is particularly important for plant seed germination (Figure 6.76), since the seedling is not exposed to sunlight. With the glyoxylate cycle, seeds can make glucose from stored lipids.

Because animals do not run the glyoxylate cycle, they cannot produce glucose from acetyl-CoA in net amounts, but plants and bacteria can. As a result, plants and bacteria can turn acetyl-CoA from fat into glucose, while animals can't. Bypassing the

oxidative decarboxylations (and substrate level phosphorylation) has energy costs, but, there are also benefits. Each turn of the glyoxylate cycle produces one FADH2 and one NADH instead of the three NADHs, one FADH2, and one GTP made in each turn of the citric acid cycle.



Figure 6.76 - A gingko seed embryo. Wikipedia

Carbohydrate needs

Organisms that make cell walls, such as plants, fungi, and bacteria, need large quantities of carbohydrates as they grow to support the biosynthesis of the complex structural polysaccharides of the walls. These include cellulose, glucans, and chitin. Notably, each of the organisms can operate the glyoxylate cycle using acetyl-CoA from β-oxidation.

Coordination of the glyoxylate cycle and the citric acid cycle

The citric acid cycle is a major catabolic pathway producing a considerable amount of energy for cells, whereas the glyoxylate cycle's main function is anabolic - to allow production of glucose from fatty acids in plants and bacteria. The two pathways are physically separated from each other (glyoxylate cycle in glyoxysomes / citric acid cycle in mitochondria), but nonetheless a coordinated regulation of them is important.

The enzyme that appears to provide controls for the cycle is isocitrate dehydrogenase. In plants and bacteria, the enzyme can be inactivated by phosphorylation by a kinase found only in those cells. Inactivation causes isocitrate to accumulate in the mitochondrion and when this happens, it gets shunted to the glyoxysomes, favoring the glyoxylate cycle. Removal of the phosphate from isocitrate dehydrogenase is catalyzed by an isocitrate dehydrognease-specific phosphoprotein phosphatase and restores activity to the enzyme.

When this happens, isocitrate oxidation resumes in the mitochondrion along with the rest of the citric acid cycle reactions. In bacteria, where the enzymes for both cycles are present together in the cytoplasm, accumulation of citric acid cycle intermediates and glycolysis intermediates will tend to favor the citric acid cycle by activating the phosphatase, whereas high energy conditions will tend to favor the glyoxylate cycle by inhibiting it.

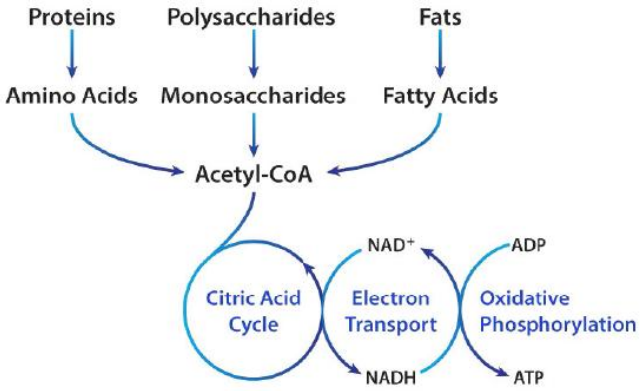


Figure 6.77 - Acetyl-CoA metabolism. Image by Aleia Kim

Acetyl-CoA metabolism

Acetyl-CoA is one of the most "connected" metabolites in biochemistry, appearing in fatty acid oxidation/synthesis, pyruvate oxidation, the citric acid cycle, amino acid anabolism/catabolism, ketone body metabolism, steroid/bile acid synthesis, and (by

extension from fatty acid metabolism) prostaglandin synthesis . Most of these pathways will be dealt with separately. Here we will cover ketone body metabolism.

Ketone body metabolism

Ketone bodies are molecules made when the blood levels of glucose fall very low. Ketone bodies can be converted to acetyl-CoA by reversing the reaction of the pathway that makes them (Figure 6.78). Acetyl CoA, of course, can be used for ATP synthesis via the citric acid cycle. People who are very hypoglycemic (including some diabetics) will produce ketone bodies (Figure 6.79) and these are often first detected by the smell of acetone on their breath.

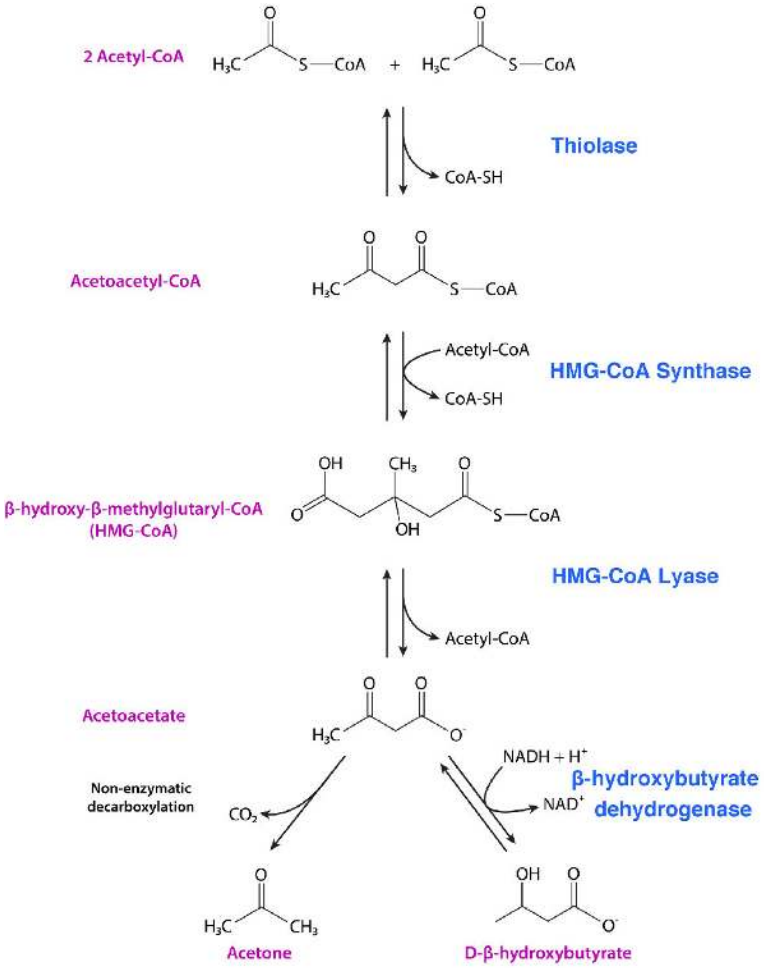


Figure 6.78 Ketone body metabolism. Image by Pehr Jacobson

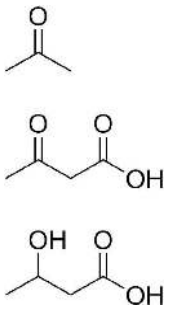


Figure 6.79 - Three ketone bodies - acetone (top), acetoacetic acid (middle), and β-hydroxybutyrate (bottom)

Overlapping pathways

The pathways for ketone body synthesis and cholesterol biosynthesis (Figure 6.80 and see HERE) overlap at the beginning. Each of these starts by combining two acetyl-CoAs together to make acetoacetyl-CoA. Not coincidentally, that is the next to last product of β-oxidation of fatty acids with even numbers of carbons (see HERE for fatty acid oxidation). In fact, the enzyme that catalyzes the joining is the same as the one that catalyzes its breakage in fatty acid oxidation – thiolase. Thus, these pathways start by reversing the last step of the last round of fatty acid oxidation.

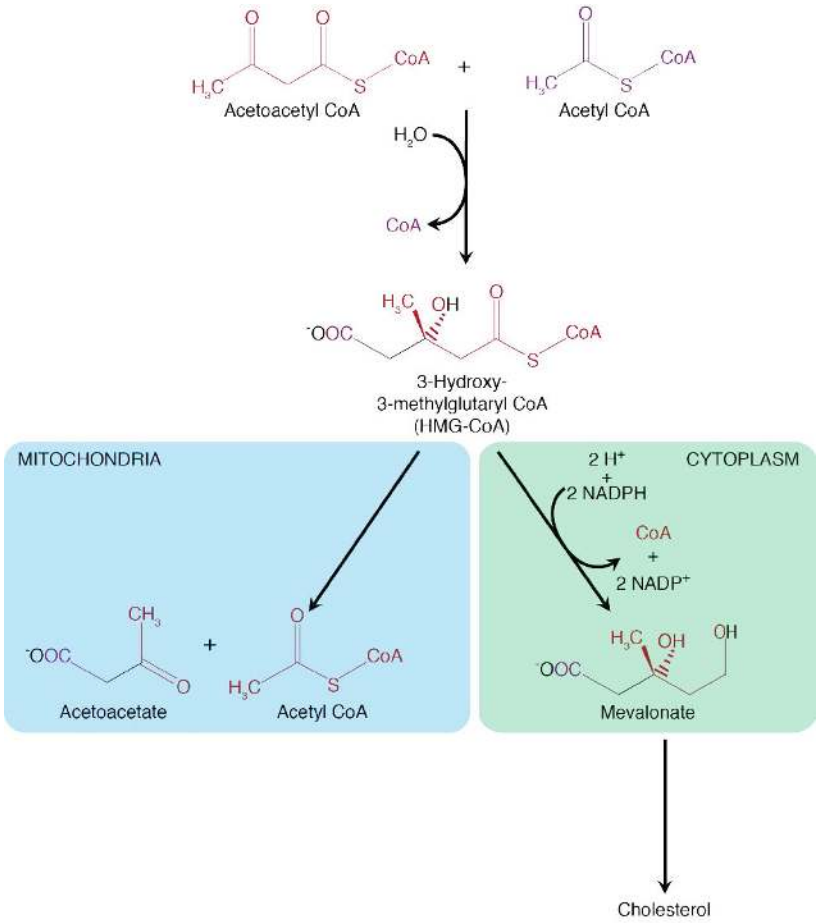


Figure 6.80 - Diverging biosynthetic pathways for ketone bodies (left) and cholesterol biosynthesis (right). Image by Penelope Irving

HMG-CoA formation

Both pathways also include addition of two more carbons to acetoacetyl-CoA from a third acetyl-CoA to form hydroxy-methyl-glutaryl-CoA, or HMG-CoA, as it is more commonly known. It is at this point that the two pathways diverge. HMG-CoA is a branch point between the two pathway and can either go on to become cholesterol or ketone bodies. In the latter pathway, HMG-CoA is broken down into acetyl-CoA and acetoacetate.

Acetoacetate is itself a ketone body and can be reduced to form another one, D-β-hydroxybutyrate (not actually a ketone, though). Alternatively, acetoacetate can be converted into acetone. This latter reaction can occur either spontaneously or via catalysis by acetoacetate decarboxylase. Acetone can be converted into pyruvate and pyruvate can be made into glucose.

D-β-hydroxybutyrate travels readily in the blood and crosses the blood-brain barrier. It can be oxidized back to acetoacetate, converted to acetoacetyl-CoA, and then broken down to two molecules of acetyl-CoA for oxidation in the citric acid cycle.

Ketosis

When a body is producing ketone bodies for its energy, this state in the body is known as ketosis. Formation of ketone bodies in the liver is critical. Normally glucose is the body's primary energy source. It comes from the diet, from the breakdown of storage

carbohydrates, such as glycogen, or from glucose synthesis (gluconeogenesis). Since the primary stores of glycogen are in muscles and liver and since gluconeogenesis occurs only in liver, kidney, and gametes, when the supply of glucose is interrupted for any reason, the liver must supply an alternate energy source.

From fatty acid breakdown

In contrast to glucose, ketone bodies can be made in animals from the breakdown of fat/fatty acids. Most cells of the body can use ketone bodies as energy sources. Ketosis may arise from fasting, a very low carbohydrate diet or, in some cases, diabetes.

Acidosis

The term acidosis refers to conditions in the body where the pH of arterial blood drops below 7.35. It is the opposite of the condition of alkalosis, where the pH of the arterial blood rises above 7.45. Normally, the pH of the blood stays in this narrow pH range. pH values of the blood lower than 6.8 or higher than 7.8 can cause irreversible damage and may be fatal. Acidosis may have roots in metabolism (metabolic acidosis) or in respiration (respiratory acidosis).

There are several causes of acidosis. In metabolic acidosis, production of excess lactic acid or failure of the kidneys to excrete acid can cause blood pH to drop. Lactic acid is produced in the body when oxygen is limiting, so anything that interferes with oxygen delivery may create conditions favoring production of excess lactic acid. These may include restrictions in the movement of blood to target tissues, resulting in hypoxia (low oxygen conditions) or decreases in blood volume. Issues with blood movement can result from heart problems, low blood pressure, or hemorrhaging.

Strenuous exercise can also result in production of lactic acid due to the inability of the blood supply to deliver oxygen as fast as tissues require it (hypovolemic shock). At the end of the exercise, though, the oxygen supply via the blood system quickly catches up.

Respiratory acidosis arises from accumulation of carbon dioxide in the blood. Causes include hypoventilation, pulmonary problems, emphysema, asthma, and severe pneumonia.

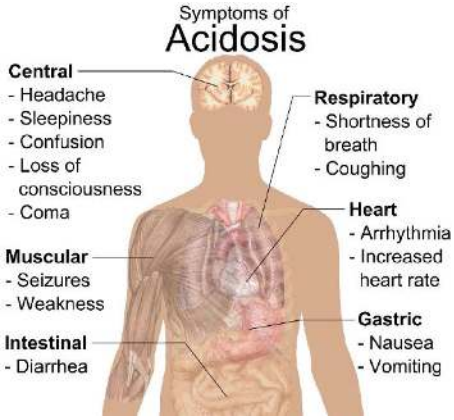


Figure 6.81 - Symptoms of acidosis

This page titled 6.3: Citric Acid Cycle and Related Pathways is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **6.2: Citric Acid Cycle & Related Pathways** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

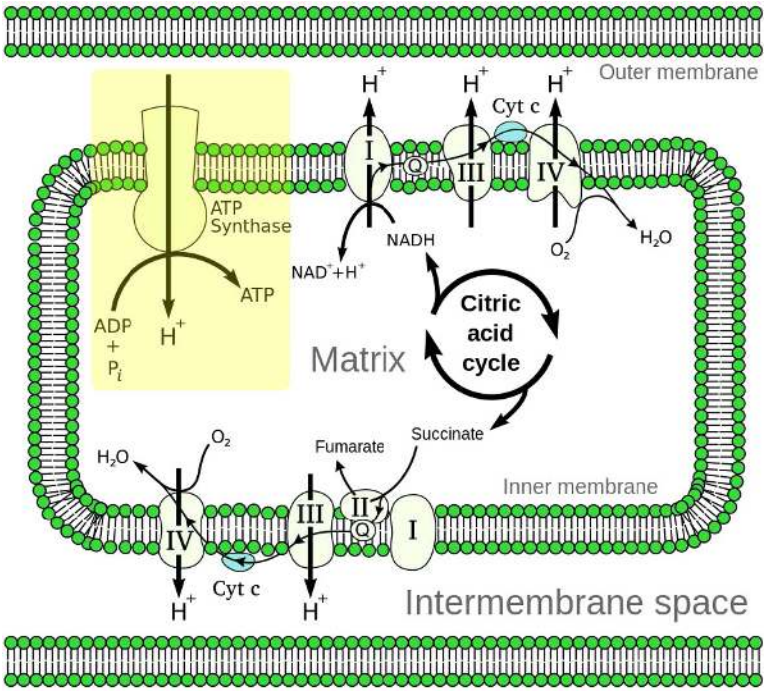
6.4: Electron Transport and Oxidative Phosphorylation

Source: BiochemFFA_5_2.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy



In eukaryotic cells, the vast majority of ATP synthesis occurs in the mitochondria in a process called oxidative phosphorylation. Even plants, which generate ATP by photophosphorylation in chloroplasts, contain mitochondria for the synthesis of ATP through oxidative phosphorylation.

Oxidative phosphorylation is linked to a process known as electron transport (Figure 5.14). The electron transport system, located in the inner mitochondrial membrane, transfers electrons donated by the reduced electron carriers NADH and FADH2 (obtained from glycolysis, the citric acid cycle or fatty acid oxidation) through a series of electrons acceptors, to oxygen. As we shall see, movement of electrons through complexes of the electron transport system essentially "charges" a battery that is used to make ATP in oxidative phosphorylation. In this way, the oxidation of sugars and fatty acids is coupled to the synthesis of ATP, effectively extracting energy from food.



Chemiosmotic model

Dr. Peter Mitchell introduced a radical proposal in 1961 to explain the mechanism by which mitochondria make ATP. It is known as the chemiosmotic hypothesis and has been shown over the years to be correct. Mitchell proposed that synthesis of ATP in mitochondria depends on an electrochemical gradient, across the mitochondrial inner membrane, that arises ultimately from the energy of reduced electron carriers, NADH and FADH2.

Electron transport

Further, the proposal states that the gradient is created when NADH and FADH2 transfer their electrons to an electron transport system (ETS) located in the inner mitochondrial membrane. Movement of electrons through a series of of electron carriers is coupled to the pumping of protons out of the mitochondrial matrix across the inner mitochondrial membrane into the space between the inner and outer membranes. The result is creation of a gradient of protons whose potential energy can be used to make ATP. Electrons combine with oxygen and protons at the end of the ETS to make water.

ATP synthase

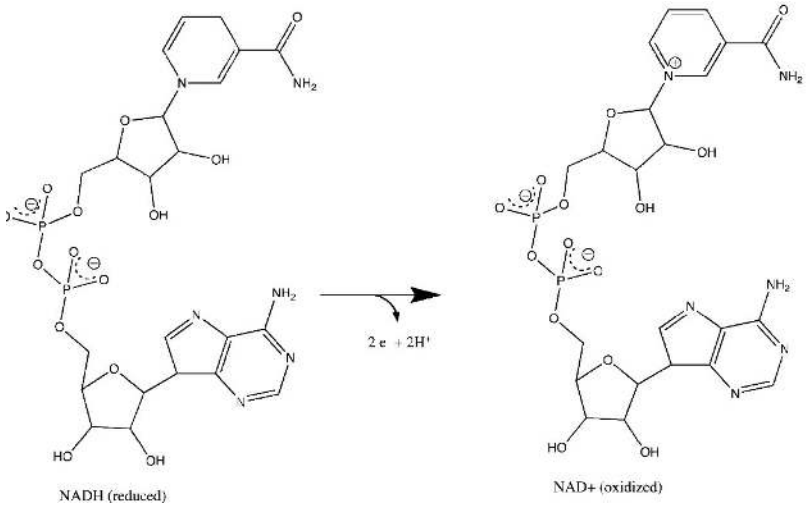
In oxidative phosphorylation, ATP synthesis is accomplished as a result of protons re-entering the mitochondrial matrix via the transmembrane ATP synthase complex, which combines ADP with inorganic phosphate to make ATP. Central to the proper functioning of mitochondria through this process is the presence of an intact mitochondrial inner membrane impermeable to protons.

Tight coupling

When this is the case, tight coupling is said to exist between electron transport and the synthesis of ATP (called oxidative phosphorylation). Chemicals which permeabilize the inner mitochondrial membrane to protons cause uncoupling, that is, they allow the protons to leak back into the mitochondrial matrix, rather than through the ATP synthase, so that the movement of electrons through the ETS is no longer linked to the synthesis of ATP.

Power plants

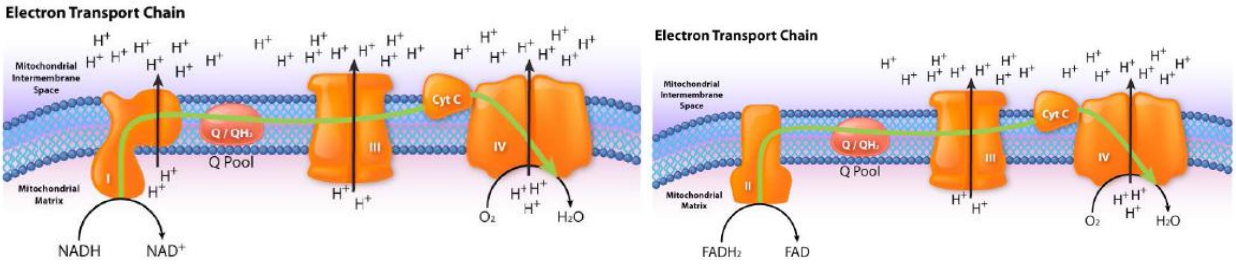
Mitochondria are called the power plants of the cell because most of a cell's ATP is produced there in the process of oxidative phosphorylation. The mechanism by which ATP is made in oxidative phosphorylation is one of the most interesting in all of biology.



Considerations

The process has three primary considerations. The first is electrical – electrons from reduced electron carriers, such as NADH and FADH2, enter the electron transport system via Complex I and II, respectively. As seen in Figure 5.16 and Figure 5.17, electrons move from one complex to the next, not unlike the way they move through an electrical circuit. Such movement occurs a a result of

a set of reduction-oxidation (redox) reactions with electrons moving from a more negative reduction potential to a more positive one.



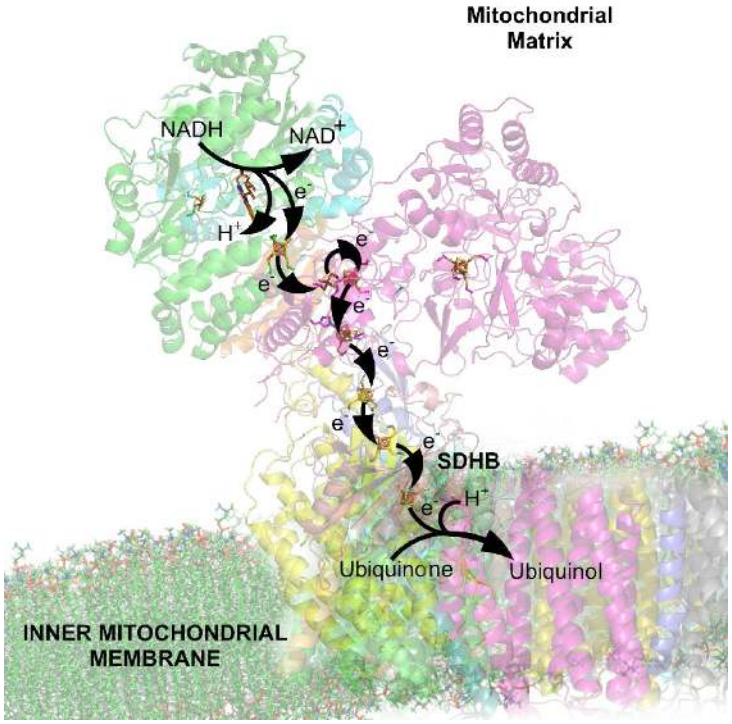
One can think of this occurring as a process where carriers “take” electrons away from complexes with lower reduction potential, much the way a bully takes lunch money from a smaller child. In this scheme, the biggest “bully” is oxygen in Complex IV. Electrons gained by a carrier cause it to be reduced, whereas the carrier giving up the electrons is oxidized.

Entry of electrons to system

Movement of electrons through the chain begins either by 1) transfer from NADH to Complex I (Figure 5.16) or 2) movement of electrons through a covalently bound FADH2 (Figure 5.17) in the membrane-bound succinate dehydrogenase (Complex II). (An alternate entry point for electrons from FADH2 is the Electron Transferring Flavoprotein via the electron-transferring-flavoprotein dehydrogenase, not shown).

Traffic cop

Both Complex I and II pass electrons to the inner membrane’s coenzyme Q (CoQ - Figures 5.18 & 5.19). In each case, coenzyme Q accepts electrons in pairs and passes them off to Complex III (CoQH2-cytochrome c reductase) singly. Coenzyme Q thus acts as a traffic cop, regulating the flow of electrons through the ETS.



Docking station

Complex III is a docking station or interchange for the incoming electron carrier (coenzyme Q) and the outgoing carrier (cytochrome c). Movement of electrons from Coenzyme Q to Complex III and then to cytochrome C occurs as a result of what is

referred to as the Q-cycle (see below).

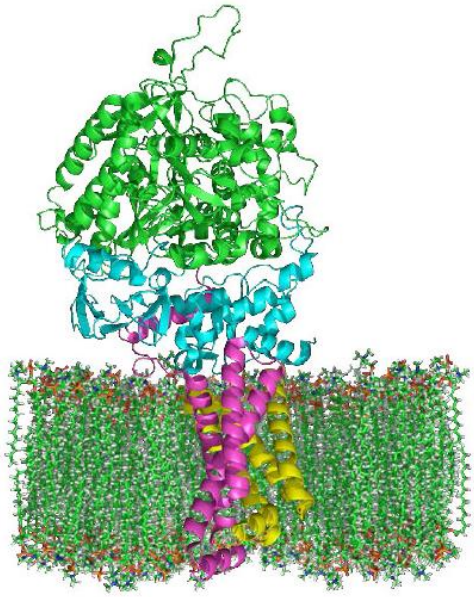
Complex III acts to ferry electrons from CoQ to cytochrome c. Cytochrome c takes one electron from Complex III and passes it to Complex IV (cytochrome oxidase). Complex IV is the final protein recipient of the electrons. It passes them to molecular oxygen (O2) to make two molecules of water. Making two water molecules requires four electrons, so Complex IV must accept, handle, and pass to molecular oxygen four separate electrons, causing the oxidation state of oxygen to be sequentially changed with addition of each electron.

Proton pumping

As electrons pass through complexes I, III, and IV, there is a release of a small amount of energy at each step, which is used to pump protons from the mitochondrial matrix (inside of mitochondrion) and deposit them in the intermembrane space (between the inner and outer membranes of the mitochondrion). The effect of this redistribution is to increase the electrical and chemical potential across the membrane.

Potential energy

As discussed earlier, electrochemical gradients have potential energy. Students may think of the process as “charging the battery.” Just like a charged battery, the potential arising from the proton differential across the membrane can be used to do things. In the mitochondrion, what the proton gradient does is facilitate the production of ATP from ADP and Pi. This process is known as oxidative phosphorylation, because the phosphorylation of ADP to ATP is dependent on the oxidative reactions occurring in the mitochondria.



Having understood the overall picture of the synthesis of ATP linked to the movement of electrons through the ETS, we will take a closer look at the individual components of the ETS.

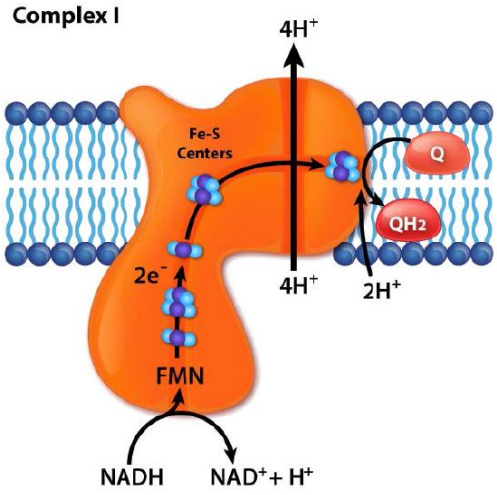
Complex I

Complex I (also called NADH:ubiquinone oxidoreductase or NADH dehydrogenase (ubiquinone)) is the electron acceptor from NADH in the electron transport chain and the largest complex found in it.

Complex I contains 44 individual polypeptide chains, numerous iron-sulfur centers, a molecule of flavin mononucleotide (FMN) and has an L shape with about 60 transmembrane domains. In the process of electron transport through it, four protons are pumped across the inner membrane into the intermembrane space and electrons move from NADH to coenzyme Q, converting it from

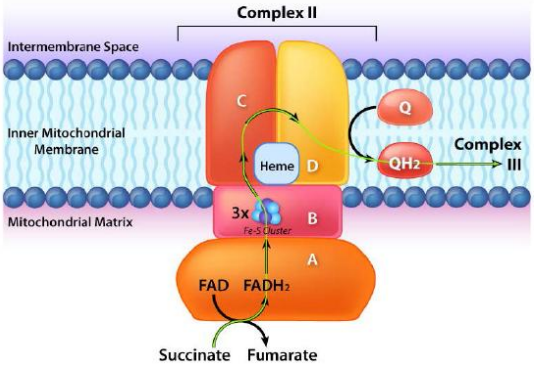
ubiquinone (no electrons) to ubiquinol (gain of two electrons). An intermediate form, ubisemiquinone (gain of one electron), is found in the Q-cycle.

Electrons travel through the complex via seven primary iron sulfur centers. The best known inhibitor of the complex, rotenone, works by binding to the CoQ binding site. Other inhibitors include ADP-ribose (binds to the NADH site) and piericidin A (rotenone analog). The process of electron transfer through complex I is reversible and when this occurs, superoxide (a reactive oxygen species) may be readily generated.



Complex II

Complex II (also called succinate dehydrogenase or succinate-coenzyme Q reductase) is a membrane bound enzyme of the citric acid cycle that plays a role in the electron transport process, transferring electrons from its covalently bound FADH2 to coenzyme Q. The process occurs, as shown in Figure 5.20 and Figure 5.21, with transfer of electrons from succinate to FAD to form FADH2 and fumarate. FADH2, in turn, donates electrons to a relay system of iron-sulfur groups and they ultimately reduce ubiquinone (CoQ) along with two protons from the matrix to ubiquinol. The role of the heme group in the process is not clear. Inhibitors of the process include carboxin, malonate, malate, and oxaloacetate. The role of citric acid cycle intermediates as inhibitors is thought to be due to inhibition of the reversal of the transfer process which can produce superoxide.

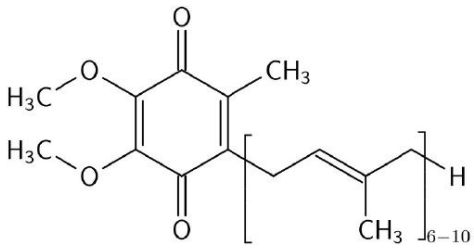


Coenzyme Q

Coenzyme Q (Figure 5.23) is a 1,4 benzoquinone whose name is often given as Coenzyme Q10, CoQ, or Q10. The 10 in the name refers to the number of isoprenyl units it contains that anchor it to the mitochondrial inner membrane. CoQ is a vitamin-like lipid substance found in most eukaryotic cells as a component of the electron transport system. The requirement for CoQ increases with increasing energy needs of cells, so the highest concentrations of CoQ in the body are found in tissues that are the most metabolically active - heart, liver, and kidney.

Three forms

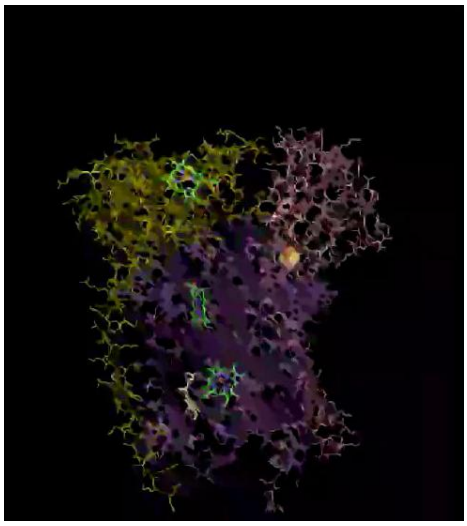
CoQ is useful because of its ability to carry and donate electrons and particularly because it can exist in forms with two extra electrons (fully reduced - ubiquinol), one extra electron (semi-reduced - ubisemiquinone), or no extra electrons (fully oxidized - ubiquinone). This ability allows CoQ to provide transition between the first part of the electron transport system that moves electrons in pairs and the last part of the system that moves electrons one at a time.

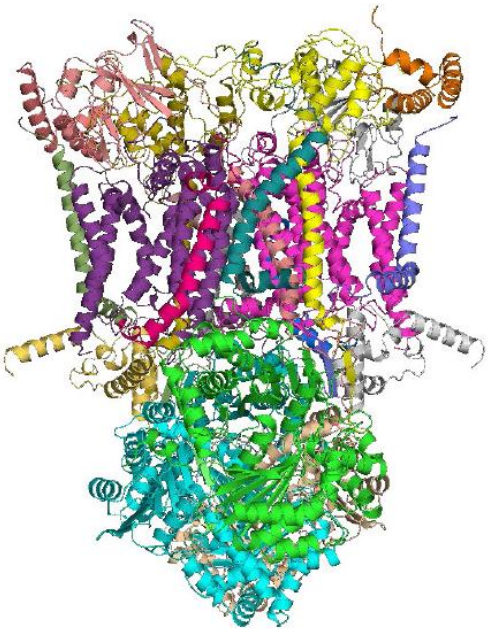


Complex III

Complex III (also known as coenzyme Q : cytochrome c — oxidoreductase or the cytochrome bc1 complex - Figure 5.24) is the third electron accepting complex of the electron transport system. It is a transmembrane protein with multiple subunits present in the mitochondria of all aerobic eukaryotic organisms and and the cell membrane of almost all bacteria. The complex contains 11 subunits, a 2-iron ferredoxin, cytochromes b and c1 and belongs to the family of oxidoreductase enzymes.

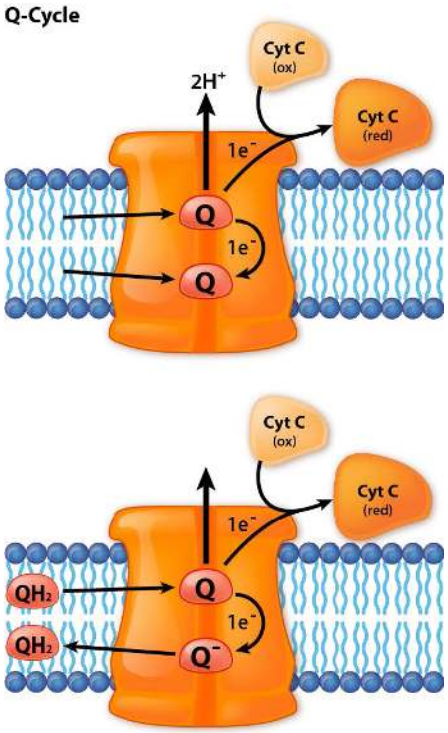
It accepts electrons from coenzyme Q in electron transport and passes them off to cytochrome c. In this cycle, known as the Q cycle, electrons arrive from CoQ in pairs, but get passed to cytochrome c individually. In the overall process, two protons are consumed from the matrix and four protons are pumped into the intermembrane space. Movement of electrons through the complex can be inhibited by antimycin A, myxothiazol, and stigmatellin. Complex III is also implicated in creation of superoxide (a reactive oxygen species) when electrons from it leak out of the chain of transfer. The phenomenon is more pronounced when antimycin A is present.





Q-cycle

In the Q-cycle, electrons are passed from ubiquinol (QH2) to cytochrome c using Complex III as an intermediary docking station for the transfer. Two pair of electrons enter from QH2 and one pair is returned to another CoQ to re-make QH2. The other pair is donated singly to two different cytochrome c molecules.



Step one

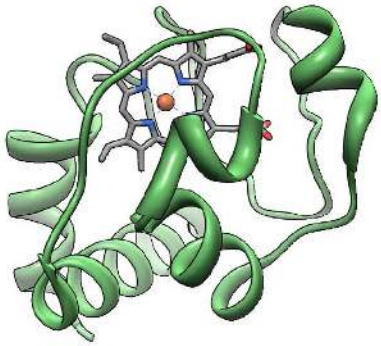
The Q-cycle happens in a two step process. First, a ubiquinol (CoQH2) and a ubiquinone (CoQ) dock at Complex III. Ubiquinol transfers two electrons to Complex III. One electron goes to a docked cytochrome c, reducing it and it exits (replaced by an oxidized cytochrome c). The other goes to the docked uniquinone to create the semi-reduced semiubiquinone (CoQ.-) and leaving behind a ubiquinone, which exits. This is the end of step 1.

Step two

The gap left behind by the ubiquinone (Q) that departed is replaced by another ubiquinol (QH2). It too donates two electrons to Complex III, which splits them. One goes to the newly docked oxidized cytochrome c, which is reduced and exits. The other goes to the ubisemiquinone. Two protons from the matrix combine with it to make another ubiquinol. It and the ubiquinone created by the electron donation exit Complex III and the process starts again. In the overall process, two protons are consumed from the matrix and four protons are pumped into the intermembrane space.

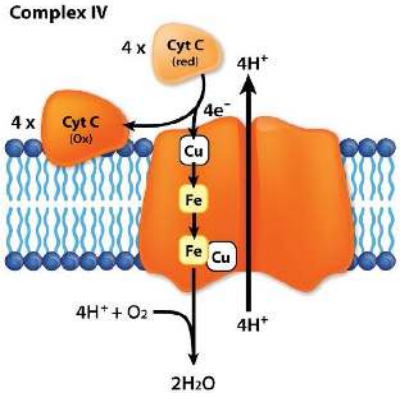
Cytochrome c

Cytochrome c (Figure 5.26) is a small (12,000 Daltons), highly conserved protein, from unicellular species to animals, that is loosely associated with the inner mitochondrial membrane where it functions in electron transport. It contains a heme group which is used to carry a single electron from Complex III to Complex IV. Cytochrome c also plays an important role in apoptosis in higher organisms. Damage to the mitochondrion that results in release of cytochrome c can stimulate assembly of the apoptosome and activation of the caspase cascade that leads to programmed cell death.



Complex IV

Complex IV, also known as cytochrome c oxidase is a 14 subunit integral membrane protein at the end of the electron transport chain (Figure 5.27). It is responsible for accepting one electron each from four cytochrome c proteins and adding them to molecular oxygen (O2) along with four protons from the mitochondrial matrix to make two molecules of water. Four protons from the matrix are also pumped into the intermembrane space in the process. The complex has two molecules of heme, two cytochromes (a and a3), and two copper centers (called CuA ad CuB). Cytochrome c docks near the CuA and donates an electron to it. The reduced CuA passes the electron to cytochrome a, which turns it over to the a3-CuB center where the oxygen is reduced. The four electrons are thought to pass through the complex rapidly resulting in complete reduction of the oxygen-oxygen molecule without formation of a peroxide intermediate or superoxide, in contrast to previous predictions.



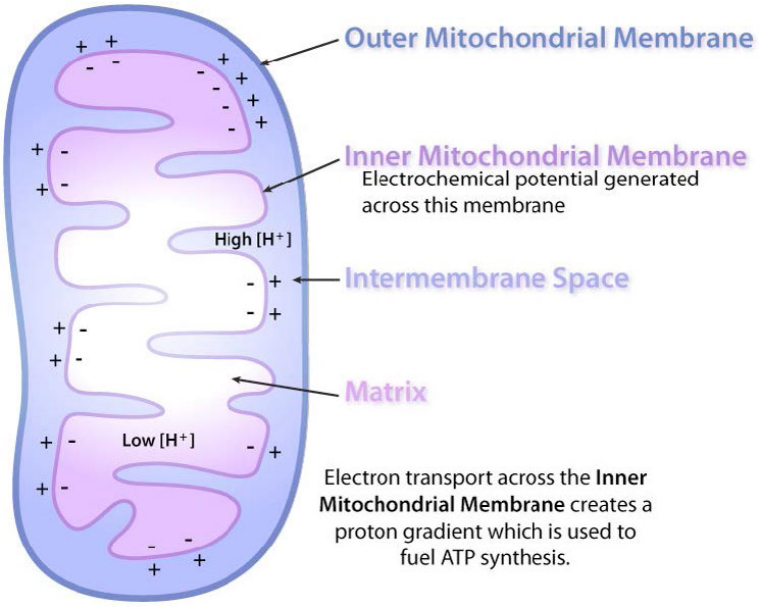
Respirasome

There has been speculation for many years that a supercomplex of electron carriers in the inner membrane of the mitochondrion may exist in cells with individual carriers making physical contact with each other. This would make for more efficient transfer

reactions, minimize the production of reactive oxygen species and be similar to metabolons of metabolic pathway enzymes, for which there is some evidence. Now, evidence appears to be accumulating that complexes I, III, and IV form a supercomplex, which has been dubbed the respirasome1.

Oxidative phosphorylation

The process of oxidative phosphorylation uses the energy of the proton gradient established by the electron transport system as a means of phosphorylating ADP to make ATP. The establishment of the proton gradient is dependent upon electron transport. If electron transport stops or if the inner mitochondrial membrane's impermeability to protons is compromised, oxidative phosphorylation will not occur because without the proton gradient to drive the ATP synthase, there will be no synthesis of ATP.



ATP synthase

The protein complex harvesting energy from the proton gradient and using it to make ATP from ADP is an enzyme that has several names - Complex V, PTAS (Proton Translocating ATP Synthase), and ATP synthase (Figure 5.29). Central to its function is the movement of protons through it (from the intermembrane space back into the matrix). Protons will only provide energy to make ATP if their concentration is greater in the intermembrane space than in the matrix and if ADP is available.

It is possible, in some cases, for the concentration of protons to be greater inside the matrix than outside of it. When this happens, the ATP synthase can run backwards, with protons moving from inside to out, accompanied by conversion of ATP to ADP + Pi. This is usually not a desirable circumstance and there are some controls to reduce its occurrence.

Normally, ATP concentration will be higher inside of the mitochondrion and ADP concentration be higher outside the mitochondrion. However, when the rate of ATP synthesis exceeds the rate of ATP usage, then ATP concentrations rise outside the mitochondrion and ADP concentrations fall everywhere.

This may happen, for example, during periods of rest. It has the overall effect of reducing transport and thus lowering the concentration of ADP inside the matrix. Reducing ADP concentration in the matrix reduces oxidative phosphorylation and has effects on respiratory control (see HERE).

Another important consideration is that when ATP is made in oxidative phosphorylation, it is released into the mitochondrial matrix, but must be transported into the cytosol to meet the energy needs of the rest of the cell. This is accomplished by action of the adenine nucleotide translocase, an antiport that moves ATP out of the matrix in exchange for ADP moving into the matrix. This transport system is driven by the concentrations of ADP and ATP and ensures that levels of ADP are maintained within the mitochondrion, permitting continued ATP synthesis.

One last requirement for synthesis of ATP from ADP is that phosphate must also be imported into the matrix. This is accomplished by action of the phosphate translocase, which is a symport that moves phosphate into the mitochondrial matrix along with a proton.

There is evidence that the two translocases and ATP synthase may exist in a complex, which has been dubbed the ATP synthasome.

In summary, the electron transport system charges the battery for oxidative phosphorylation by pumping protons out of the mitochondrion. The intact inner membrane of the mitochondrion keeps the protons out, except for those that re-enter through ATP Synthase. The ATP Synthase allows protons to re-enter the mitochondrial matrix and harvests their energy to make ATP.

ATP synthase mechanism

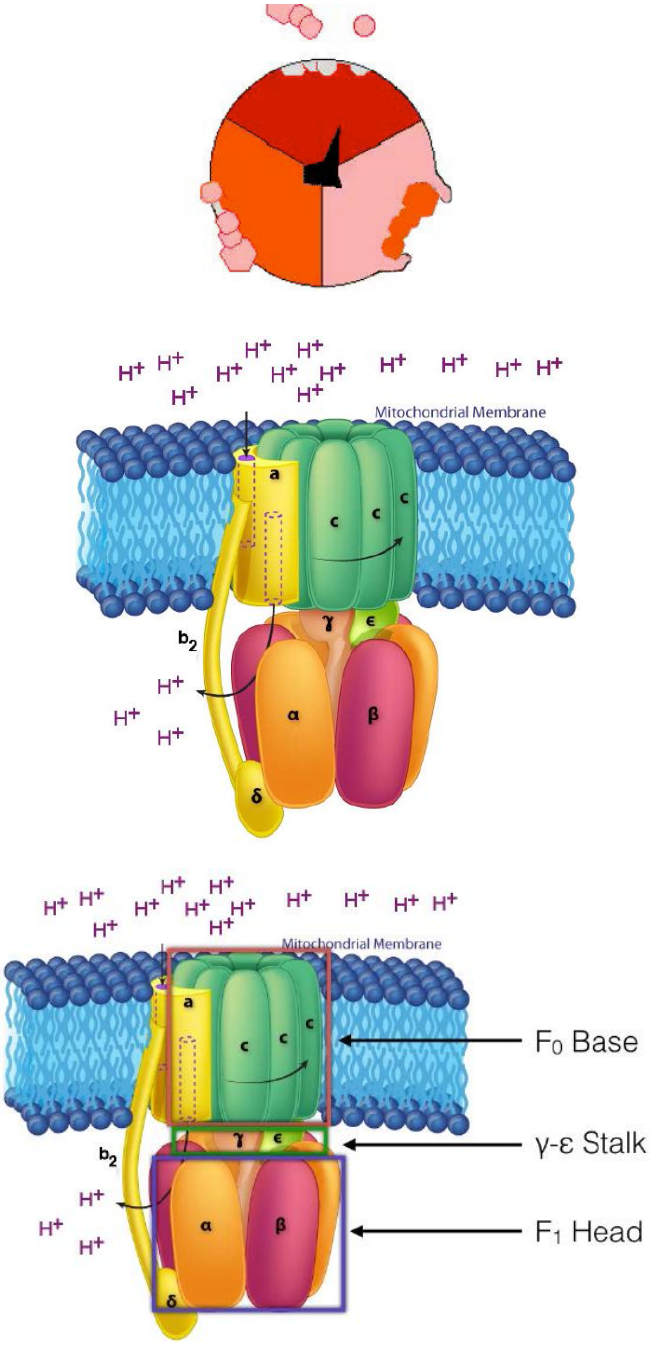


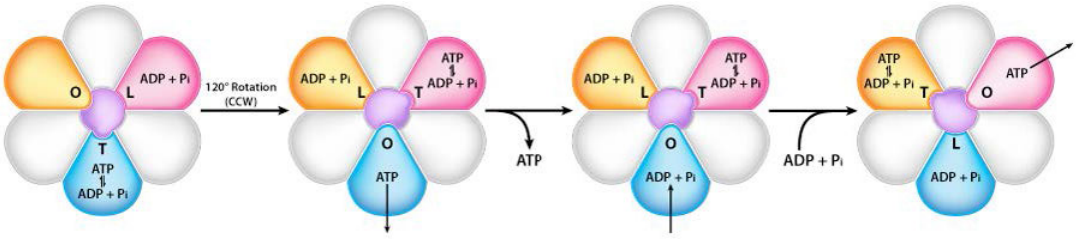
Figure 5.32 illustrate the multi-subunit nature of this membrane protein, which acts like a turbine at a hydroelectric dam. The movement of protons through the ATP Synthase c-ring causes it and the γ-ε stalk attached to it to turn. It is this action that is necessary for making ATP.

In ATP Synthase, the spinning components, or rotor, are the membrane portion (c ring) of the F0 base and the γ-ε stalk, which is connected to it. The γ-ε stalk projects into the F1 head of the mushroom structure. The F1 head contains the catalytic ability to make ATP. The F1 head is hexameric in structure with paired α and β proteins arranged in a trimer of dimers. ATP synthesis occurs within the β subunits.

Rotation of γ unit

Turning of the γ shaft (caused by proton flow) inside the α-β trimer of the F1 head causes each set of β proteins to change structure slightly into three different forms called Loose, Tight, and Open (L,T,O - Figure 5.31). Each of these forms has a function.

The Loose form binds ADP + Pi. The Tight form "squeezes" them together to form the ATP. The Open form releases the ATP into the mitochondrial matrix. Thus, as a result of the proton flow through the ATP synthase, from the intermembrane space into the matrix, ATP is made from ADP and Pi.



Respiratory control

When a mitochondrion has an intact inner membrane and protons can only return to the matrix by passing through the ATP synthase, the processes of electron transport and oxidative phosphorylation are said to be tightly coupled.

Interdependence

In simple terms, tight coupling means that the processes of electron transport and oxidative phosphorylation are interdependent. Without electron transport going on in the cell, oxidative phosphorylation will soon stop.

The reverse is also true, because if oxidative phosphorylation stops, the proton gradient will not be dissipated as it is being built by the electron transport system and will grow larger and larger. The greater the gradient, the greater the energy needed to pump protons out of the mitochondrion. Eventually, if nothing relieves the gradient, it becomes too large and the energy of electron transport is insufficient to perform the pumping. When pumping stops, so too does electron transport.

ADP dependence

Another relevant point is that ATP synthase is totally dependent upon a supply of ADP. In the absence of ADP, the ATP synthase stops functioning and when it stops, so too does movement of protons back into the mitochondrion. With this information, it is possible to understand the link between energy usage and metabolism. The root of this, as noted, is respiratory control.

At rest

To illustrate these links, let us first consider a person, initially at rest, who then suddenly jumps up and runs away. At first, the person's ATP levels are high and ADP levels are low (no exercise to burn ATP), so little oxidative phosphorylation is occurring and thus the proton gradient is high. Electron transport is moving slowly, if at all, so it is not using oxygen and the person's breathing is slow, as a result.

Exercise

When running starts, muscular contraction, which uses energy, causes ATP to be converted to ADP. Increasing ADP in muscle cells favors oxidative phosphorylation to attempt to make up for the ATP being burned. ATP synthase begins working and protons begin to come back into the mitochondrial matrix. The proton gradient decreases, so electron transport re-starts.

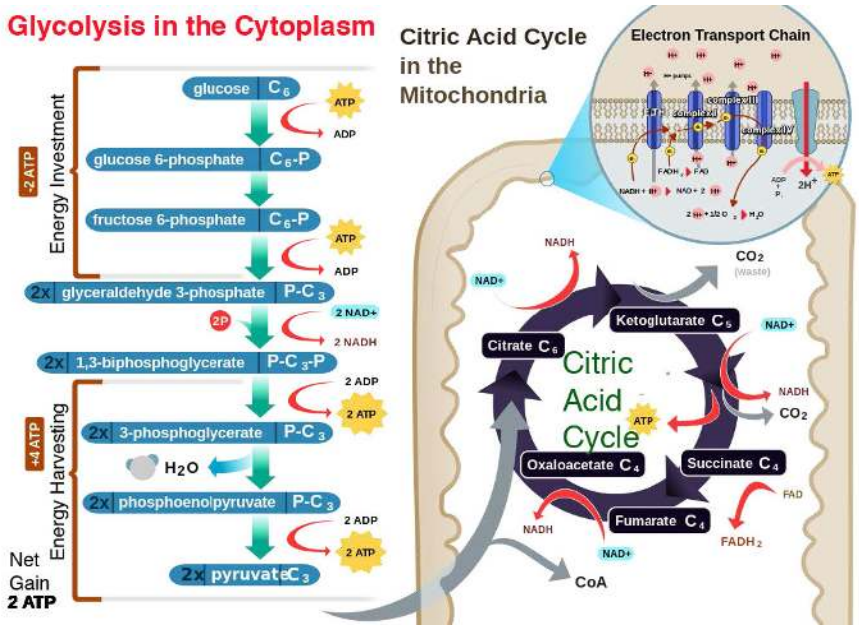
Electron transport needs an electron acceptor, so oxygen use increases and when oxygen use increases, the person starts breathing more heavily to supply it. When the person stops running, ATP concentrations get rebuilt by ATP synthase. Eventually, when ATP levels are completely restored, ADP levels fall and ATP synthase stops or slows considerably. With little or no proton movement,

electron transport stops because the proton gradient is too large. When electron transport stops, oxygen use decreases and the rate of breathing slows down.

Electron transport critical

The really interesting links to metabolism occur relative to whether or not electron transport is occurring. From the examples, we can see that electron transport will be relatively slowed when not exercising and more rapid when exercise (or other ATP usage) is occurring. Remember that electron transport is the way in which reduced electron carriers, NADH and FADH2, donate their electrons to the ETS , becoming oxidized to NAD+ and FAD, respectively.

Oxidized carriers, such as NAD+ and FAD are needed by catabolic pathways, like glycolysis, the citric acid cycle, and fatty acid oxidation. Anabolic pathways, such as fatty acid/fat synthesis and gluconeogenesis rely on reduced electron carriers, such as FADH2, NADH, and the related carrier, NADPH.



Links to exercise

High levels of NADH and FADH2 prevent catabolic pathways from operating, since NAD+ and FAD levels will be low and these are needed to accept the electrons released during catabolism by the oxidative processes.

Thanks to respiratory control, when one is exercising, NAD+ and FAD levels increase (because electron transport is running), so catabolic pathways that need NAD+ and FAD can function. The electrons lost in the oxidation reactions of catabolism are captured by NAD+ and FAD to yield NADH and FADH2, which then supply electrons to the electron transport system and oxidative phosphorylation to make more needed ATP.

Thus, during exercise, cells move to a mode of quickly cycling between reduced electron carriers (NADH/FADH2) and oxidized electron carriers (NAD+/FAD). This allows rapidly metabolizing tissues to transfer electrons to NAD+/FAD and it allows the reduced electron carriers to rapidly become oxidized, allowing the cell to produce ATP.

Rest

When exercise stops, NADH and FADH2 levels rise (because electron transport is slowing) causing catabolic pathways to slow/stop. If one does not have the proper amount of exercise, reduced carriers remain high in concentration for long periods of time. This means we have an excess of energy and then anabolic pathways, particularly fatty acid synthesis, are favored, so we get fatter.

Altering respiratory control

One might suspect that altering respiratory control could have some very dire consequences and that would be correct. Alterations can take the form of either inhibiting electron transport/oxidative phosphorylation or uncoupling the two . These alterations can be achieved using compounds with specific effects on particular components of the system.

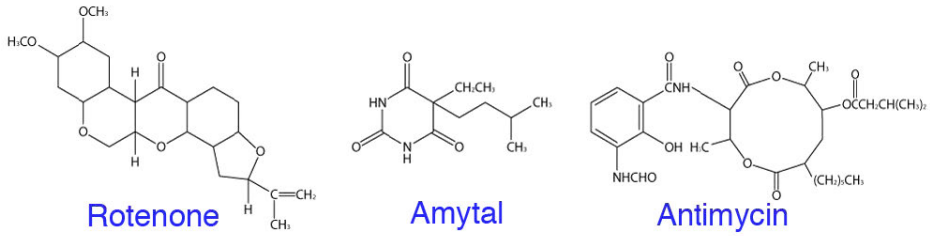
All of the chemicals described here are laboratory tools and should never be used by people. The first group for discussion are the inhibitors. In tightly coupled mitochondria, inhibiting either electron transport or oxidative phosphorylation has the effect of inhibiting the other one as well.

Electron transport inhibitors

Common inhibitors of electron transport include rotenone and amytal, which stop movement of electrons past Complex I, malonate, malate, and oxaloacetate, which inhibit movement of electrons through Complex II, antimycin A which stops movement of electrons past Complex III, and cyanide, carbon monoxide, azide, and hydrogen sulfide, which inhibit electron movement through Complex IV (Figure 5.33). All of these compounds can stop electron transport directly (no movement of electrons) and oxidative phosphorylation indirectly (proton gradient will dissipate). While some of these compounds are not commonly known, almost everyone is aware of the hazards of carbon monoxide and cyanide, both of which can be lethal.

ATP synthase inhibitor

It is also possible to use an inhibitor of ATP synthase to stop oxidative phosphorylation directly (no ATP production) and electron transport indirectly (proton gradient not relieved so it becomes increasingly difficult to pump protons out of matrix). Oligomycin A (Figure 5.34) is an inhibitor of ATP synthase.



Rotenone

Rotenone, which is a plant product, is used as a natural insecticide that is permitted for organic farming. When mitochondria are treated with this, electron transport will stop at Complex I and so, too, will the pumping of protons out of the matrix. When this occurs, the proton gradient rapidly dissipates, stopping oxidative phosphorylation as a consequence. There are other entry points for electrons than Complex I, so this type of inhibition is not as serious as using inhibitors of Complex IV, since no alternative route for electrons is available. It is for this reason that cyanide, for example, is so poisonous.

2,4-DNP

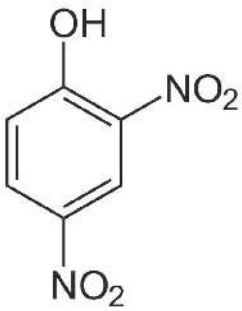


Figure 5.35). Treatment of mitochondria with 2,4 DNP makes the mitochondrial inner membrane “leaky” to protons. This has the effect of providing an alternate route for protons to reenter the matrix besides going through ATP synthase, and uncouples oxidative phosphorylation from electron transport.

Imagine a dam holding back water with a turbine generating electricity through which water must flow. When all water flows through the turbine, the maximum amount of electricity can be generated. If one pokes a hole in the dam, though, water will flow

through the hole and less electricity will be created. The generation of electricity will thus be uncoupled from the flow of water. If the hole is big enough, the water will all drain out through the hole and no electricity will be made.

Bypassing ATP synthase

Imagine, now, that the proton gradient is the equivalent of the water, the inner membrane is the equivalent of the dam and the ATP synthase is the turbine. When protons have an alternate route, little or no ATP will be made because protons will pass through the membrane's holes instead of spinning the turbine of ATP synthase.

It is important to recognize, though, that uncoupling by 2,4 DNP works differently from the electron transport inhibitors or the ATP synthase inhibitor. In those situations, stopping oxidative phosphorylation resulted in indirectly stopping electron transport, since the two processes were coupled and the inhibitors did not uncouple them. Similarly, stopping electron transport indirectly stopped oxidative phosphorylation for the same reason.

Such is not the case with 2,4 DNP. Stopping oxidative phosphorylation by destroying the proton gradient allows electron transport to continue unabated (it actually stimulates it), since the proton gradient cannot build no matter how much electron transport runs. Consequently, electron transport runs like crazy but oxidative phosphorylation stops. When that happens, NAD+ and FAD levels rise, and catabolic pathways run unabated with abundant supplies of these electron acceptors. The reason such a scenario is dangerous is because the body is using all of its nutrient resources, but no ATP is being made. Lack of ATP leads to cellular (and organismal) death. In addition, the large amounts of heat generated can raise the temperature of the body to unsafe levels.



Thermogenin

One of the byproducts of uncoupling electron transport is the production of heat. The faster metabolic pathways run, the more heat is generated as a byproduct. Since 2,4 DNP causes metabolism to speed up, a considerable amount of heat can be produced. Controlled uncoupling is actually used by the body in special tissues called brown fat. In this case, brown fat cells use the heat created to help thermoregulate the temperature of newborn children.

Permeabilization of the inner membrane is accomplished in brown fat by the synthesis of a protein called thermogenin (also known as uncoupling protein). Thermogenin binds to the inner membrane and allows protons to pass through it, thus bypassing the ATP synthase. As noted for 2,4 DNP, this results in activation of catabolic pathways and the more catabolism occurs, the more heat is generated.

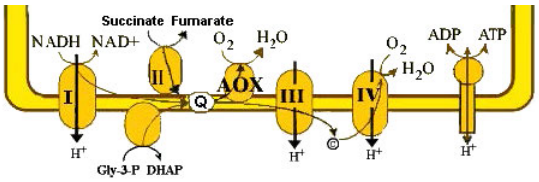
Dangerous drug

In uncoupling, whether through the action of an endogenous uncoupling protein or DNP, the energy that would have normally been captured in ATP is lost as heat. In the case of uncoupling by thermogenin, this serves the important purpose of keeping newborn infants warm. But in adults, uncoupling merely wastes the energy that would have been harvested as ATP. In other words, it mimics starvation, even though there is plenty of food, because the energy is dissipated as heat.

This fact, and the associated increase in metabolic rate, led to DNP being used as a weight loss drug in the 1930s. Touted as an effortless way to lose weight without having to eat less or exercise more, it was hailed as a magic weight loss pill. It quickly became apparent, however, that this was very dangerous. Many people died from using this drug before laws were passed to ban the use of DNP as a weight loss aid.

Alternative oxidase

Another approach to generating heat that doesn't involve breaking respiratory control is taken by some fungi, plants, and protozoa. They use an alternative electron transport. In these organisms, there is an enzyme called alternative oxidase (Figure 5.36). Alternative oxidase is able to accept electrons from CoQ and pass them directly to oxygen.



The process occurs in coupled mitochondria. Its mechanism of action is to reduce the yield of ATP, since fewer protons are being pumped per reduced electron carrier. Thus NAD+ concentrations increase, oxygen consumption increases, and the efficiency of ATP production decreases.

Organisms using this method must activate catabolic pathways by the increase in NAD+ concentration. This, in turn produces quantities of NADH and FADH2 necessary to make sufficient amounts of ATP. The byproduct of this increased catabolism is more heat. Not surprisingly, the alternative oxidase pathway can be activated by cold temperatures.

Energy efficiency

Cells are not 100% efficient in energy use. Nothing we know is. Consequently, cells do not get as much energy out of catabolic processes as they put into anabolic processes. A good example is the synthesis and breakdown of glucose, something liver cells are frequently doing. The complete conversion of glucose to pyruvate in glycolysis (catabolism) yields two pyruvates plus 2 NADH plus 2 ATPs. Conversely, the complete conversion of two pyruvates into glucose by gluconeogenesis (anabolism) requires 4 ATPs, 2 NADH, and 2 GTPs. Since the energy of GTP is essentially equal to that of ATP, gluconeogenesis requires a net of 4 ATPs more than glycolysis yields. This difference must be made up in order for the organism to meet its energy needs. It is for this reason that we eat. In addition, the inefficiency of our capture of energy in reactions results in the production of heat and helps to keep us warm, as noted. You can read more about glycolysis (HERE) and gluconeogenesis (HERE).

Metabolic controls of energy

It is also noteworthy that cells do not usually have both catabolic and anabolic processes for the same molecules occurring simultaneously inside of them (for example, breakdown of glucose and synthesis of glucose) because the cell would see no net production of anything but heat and a loss of ATPs with each turn of the cycle. Such cycles are called futile cycles and cells have controls in place to limit the extent to which they occur. Since futile cycles can, in fact, yield heat, they are used as sources of heat in some types of tissue. Brown adipose tissue of mammals uses this strategy, as described earlier. See also HERE for more on heat generation with a futile cycle.

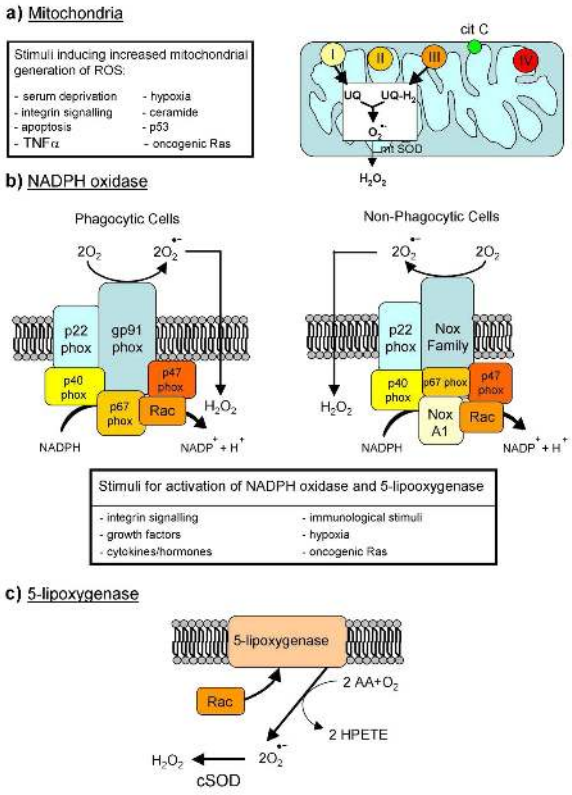


Figure 5.37) are oxygen containing molecules, such as peroxides, hydroxyl radical, superoxide, peroxynitrite, and others that are very chemically reactive. Though some ROS, such as peroxide and nitric oxide have important biological functions in signaling, increases in reactive oxygen species in times of stress can cause significant damage in the cell. Exogenous sources of ROS, such as pollution, tobacco, smoke, radiation or drugs can also cause significant problems.

Endogenous production of ROS is directed towards intracellular signaling (H2O2 and nitric oxide, for example) and defense. Many cells, for example, have NADPH oxidase (Figure 5.38) embedded in the exterior portion of the plasma membranes, in peroxisomes, and endoplasmic reticulum. It produces superoxides in the reaction below to kill bacteria .

In the immune system, cells called phagocytes engulf foreign cells and then use ROS to kill them. ROS can serve as signals for action. In zebrafish, damaged tissues have increased levels of H2O2 and this is thought to be a signal for white blood cells to converge on the site. In fish lacking the genes to produce hydrogen peroxide, white blood cells do not converge at the damage site. Sources of hydrogen peroxide include peroxisomes, which generate it as a byproduct of oxidation of long chain fatty acids.

Aging

Reactive oxygen species are at the heart of the free radical theory of aging, which states that organisms age due to the accumulation of damage from free radicals in their cells. In yeast and Drosophila, there is evidence that reducing oxidative damage can increase lifespan. In mice, increasing oxidative damage decreases life span, though in Caenorhabditis, blocking production of superoxide dismutase actually increases lifespan, so the role of ROS in aging is not completely clear.

It is clear, though, that accumulation of mitochondrial damage is problematic for individual cells. Bcl-2 proteins on the surface of mitochondria monitor damage and if they detect it, will activate proteins called Bax to stimulate the release of cytochrome c from the mitochondrial membrane, stimulating apoptosis (programmed cell death). Eventually the dead cell will be phagocytosed.

A common endogenous source of superoxide is the electron transport chain. Superoxide can be produced when movement of electrons into and out of the chain don't match well. Under these circumstances, semi-reduced CoQ can donate an electron to O2 to form superoxide (O2-). Superoxide can react with many molecules, including DNA where it can cause damage leading to mutation.

If it reacts with the aconitase enzyme, ferrous iron (Fe++) can be released which, in turn, can react in the Fenton reaction to produce another reactive oxygen species, the hydroxyl radical (Figure 5.39) .

Countering the effects of ROS are enzymes, such as catalase, superoxide dismutase, and anti-oxidants, such as glutathione and vitamins C and E.

Glutathione protects against oxidative damage by being a substrate for the enzyme glutathione peroxidase. Glutathione peroxidase catalyzes the conversion of hydrogen peroxide to water (next page).

Catalase

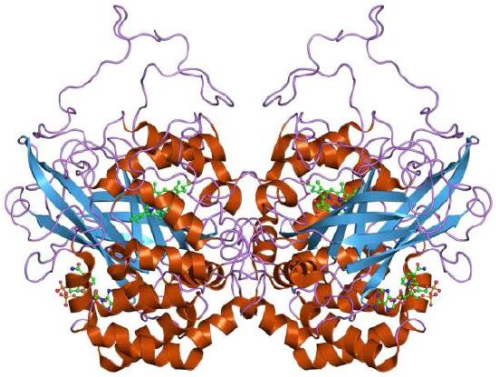


Figure 5.40) is an important enzyme for cells of all types that live in an oxygen environment. A first line of defense against reactive oxygen species, catalase catalyzes the breakdown of hydrogen peroxide into water and oxygen.

2 H2O2 <=> 2 H2O + O2

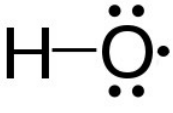
The enzyme, which employs four heme groups in its catalysis, works extremely rapidly, converting up to 40,000,000 molecules of hydrogen peroxide to water and oxygen per enzyme per second. It is abundantly found in peroxisomes.

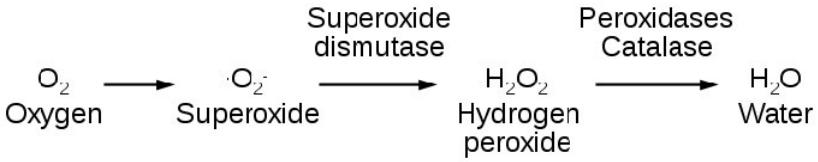
In addition to catalase's ability to break down hydrogen peroxide, the enzyme can also use hydrogen peroxide to oxidize a wide variety organic compounds, including phenols, formic acid, formaldehyde, acetaldehyde, and alcohols, but with much lower efficiency.

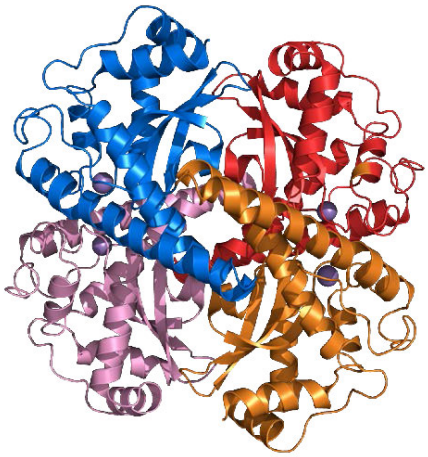
Health

The importance of catalase for health is uncertain. Mice deficient in the enzyme appear healthy and humans with low levels of the enzyme display few problems. On the other hand, mice engineered to produce higher levels of catalase, in at least one study, lived longer. The ability of organisms to live with lower levels or no catalase may arise from another group of enzymes, the peroxiredoxins, which also act on hydrogen peroxide and may make up for lower quantities of catalase. Last, there is evidence that reduced levels of catalase with aging may be responsible for the graying of hair. Higher levels of H2O2 with reduced catalase results in a bleaching of hair follicles.

Superoxide dismutase



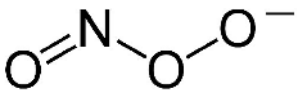




Another important enzyme for protection against reactive oxygen species is superoxide dismutase (SOD), which is found, like catalase, in virtually all organisms living in an oxygen environment. Superoxide dismutase, also like catalase, has a very high Kcat value and, in fact, has the highest Kcat/Km known for any known enzyme. It catalyzes the reactions at the top of the next column (superoxides shown in red):

The enzyme thus works by a ping-pong (double displacement) mechanism (see HERE), being converted between two different forms.

The hydrogen peroxide produced in the second reaction is easily handled by catalase and is also less harmful than superoxide, which can react with nitric oxide (NO) to form very toxic peroxynitrite ions (Figure 5.43). Peroxynitrite has negative effects on cells, as shown in Figure 5.45.



In addition to copper, an ion of Zn++ is also bound by the enzyme and likely plays a role in the catalysis. Forms of superoxide dismutase that use manganese, nickel, or iron are also known and are mostly found in prokaryotes and protists, though a manganese SOD is found in most mitochondria. Copper/zinc enzymes are common in eukaryotes.

Three forms of superoxide dismutase are found in humans and localized to the cytoplasm (SOD1 - Figure 5.45), mitochondria (SOD2 - Figure 5.46), and extracellular areas (SOD3 - Figure 5.47). Mice lacking any of the three forms of the enzyme are more sensitive to drugs, such as paraquat. Deficiency of SOD1 in mice leads to hepatocellular carcinoma and early loss of muscle tissue related to aging. Drosophila lacking SOD2 die before birth and those lacking SOD1 prematurely age.

In humans, superoxide dismutase mutations are associated with the genetically-linked form of Amyotrophic Lateral Sclerosis (ALS) and over-expression of the gene is linked to neural disorders associated with Down syndrome.

Mixed function oxidases

Other enzymes catalyzing reactions involving oxygen include the mixed function oxidases. These enzymes use molecular oxygen for two different purposes in one reaction. The mixed function part of the name is used to indicate reactions in which two different substrates are being oxidized simultaneously. Monooxygenases are examples of mixed function oxidases. An example of a mixed function oxidase reaction is shown below.

AH + BH2 + O2 <=> AOH + B + H2O

In this case, the oxygen molecule has one atom serve as an electron acceptor and the other atom is added to the AH, creating an alcohol.

Cytochrome P450 enzymes

Cytochrome P450 enzymes (called CYPs) are family of heme-containing mixed function oxidase enzymes found in all domains of life. Over 21,000 CYP enzymes are known. The most characteristic reaction catalyzed by these enzymes follows

Monooxygenase reactions such as this are relatively rare in the cell due to their use of molecular oxygen. CYPs require an electron donor for reactions like the one shown here and frequently require a protein to assist in transferring electrons to reduce the heme iron. There are six different classes of P450 enzymes based on how they get electrons

1. Bacterial P450 - electrons from ferredoxin reductase and ferredoxin

2. Mitochondrial P450 - electrons from adrenodoxin reductase and adrenodoxin

3. CYB5R/cyb5 - electrons come from cytochrome b5

4. FMN/Fd - use a fused FMN reductase

5. Microsomal P450 - NADPH electrons come via cytochrome P450 reductase or from cytochrome b5 and cytochrome b5 reductase

6. P450 only systems - do not require external reducing power

The CYP genes are abundant in humans and catalyze thousand of reactions on both cellular and extracellular chemicals. There are 57 human genes categorized into 18 different families of enzymes. Some CYPs are specific for one or a few substrates, but others can act on many different substrates.

CYP enzymes are found in most body tissues and perform important functions in synthesis of steroids (cholesterol, estrogen, testoterone, Vitamin D, e.g.), breakdown of endogenous compounds (bilirubin), and in detoxification of toxic compounds including drugs. Because they act on many drugs, changes in CYP activity can produce unexpected results and cause problems with drug interactions.

Bioactive compounds, for example, in grapefruit juice, can inhibit CYP3A4 activity, leading to increased circulating concentrations of drugs that would normally have been acted upon by CYP3A4. This is the reason that patients prescribed drugs that are known to be CYP3A4 substrates are advised to avoid drinking grapefruit juice while under treatment. St. Johns Wort, an herbal treatment, on the other hand, induces CYP3A4 activity, but inhibits CYP1A1, CYP1B1, and CYP2D6. Tobacco smoke induces CYP1A2 and watercress inhibits CYP2E1.

Cytochromes

Cytochromes are heme-containing proteins that play major roles in the process of electron transport in the mitochondrion and in photosynthesis in the chloroplast. They exist either as monomers (cytochrome c) or as subunits within large redox complexes (Complex III and Complex IV of electron transport. An atom of iron at the center of the heme group plays a central role in the process, flipping between the ferrous (Fe++) and ferric (Fe+++) states as a result of the movement of electrons through it.

There are several different cytochromes. Cytochrome c (Figure 5.47) is a soluble protein loosely associated with the mitochondrion. Cytochromes a and a3 are found in Complex IV. Complex III has cytochromes b and c1 and the plastoquinol-plastocyanin reductase of the chloroplast contains cytochromes b6 and f. Another important class of enzymes containing cytochromes is the cytochrome P450 oxidase group (see above). They get their name from the fact that they absorb light at 450 nm when their heme iron is reduced.

Iron-Sulfur Proteins

Iron-sulfur proteins contain iron-sulfur clusters in a variety of formats, including sulfide-linked di-, tri-, and tetrairon centers existing in different oxidation states (Figures 5.48 & 5.49). The clusters play a variety of roles, but the best known ones are in electron transport where they function in the redox reactions involved in the movement of electrons.

Complexes I and Complex II contain multiple Fe-S centers. Iron-sulfur proteins, though, have many other roles in cells. Aconitase uses an iron-sulfur center in its catalytic action and the ability of the enzyme to bind iron allows it to function as a barometer of iron concentration in cells. Iron-sulfur centers help to generate radicals in enzymes using S-Adenosyl Methionine (SAM) and can

serve as a source of sulfur in the synthesis of biotin and lipoic acid. Some iron-sulfur proteins even help to regulate gene expression.

Ferredoxin

Ferredoxins are iron-sulfur containing proteins performing electron transfer in a wide variety of biological systems and processes. They include roles in photosynthesis in chloroplasts. Ferredoxins are classified structurally by the iron-sulfur clustered centers they contain. Fe2S2 clusters (Figure 5.50) are found in chloroplast membranes and can donate electrons to glutamate synthase, nitrate reductase, and sulfite reductase and serve as electron carriers between reductase flavoproteins and bacterial dioxygenase systems. Adrenodoxin is a soluble human Fe2S2 ferredoxin (also called ferredoxin 1) serving as an electron carrier (to cytochrome P450) in mitochondrial monooxygenase systems. Fe4S4 ferredoxins are subdivided as low and high potential ferredoxins, with the latter ones functioning in anaerobic electron transport chains.

Ferritin

Ferritin is an intracellular iron-storage protein found in almost all living organisms, from bacteria to higher plants and animals. It is a globular protein complex with 24 subunits and is the primary intracellular iron-storage protein in eukaryotes and prokaryotes. Ferritin functions to keep iron in a soluble and non-toxic form. Its ability to safely store iron and release it in a controlled fashion allow it to act like the prime iron buffer and solubilizer in cells - keeping the concentration of free iron from going to high or falling too low. Ferritin is located in the cytoplasm in most tissues, but it is also found in the serum acting as an iron carrier. Ferritin that doesn’t contain any iron is known as apoferritin.

Monoamine oxidases

Monoamine oxidases are enzymes that catalyze the oxidative deamination of monoamines, such as serotonin, epinephrine, and dopamine. Removal of the amine with oxygen results in the production of an aldehyde and ammonia. The enzymes are found inside and outside of the central nervous system.

There are two types of monoamine oxidase enzymes - MAO-A and MAO-B. MAO-A is particularly important for oxidizing monoamines consumed in the diet. Both MAO-A and MAO-B play important roles in inactivating monoaminergic neurotransmitters. Both enzymes act on dopamine, tyramine (Figure 5.50), and tryptamine. MAO-A is the primary enzyme for metabolizing melatonin, serotonin, norepinephrine, and epinephrine, while MAO-B is the primary enzyme for phenethylamine (Figure 5.51) and benzylamine. MAO-B levels have been reported to be considerably reduced with tobacco usage.

Actions of monoamine oxidases thus affects levels of neurotransmitters and consequently are thought to play roles in neurological and/or psychiatric disorders. Aberrant levels of MAOs have been linked to numerous psychological problems, including depression, attention deficit disorder (ADD), migraines, schizophrenia, and substance abuse. Medications targeting MAOs are sometimes used to treat depression as a last resort - due to potential side effects. Excess levels of catecholamines, such as epinephrine, norepinephrine, and dopamine, can result in dangerous hypertension events.

DNA damage theory of aging

The DNA Damage Theory of Aging is based on the observation that, over time, cells are subject to extensive oxidative events. As already noted, these afford opportunities for the formation of ROS that can damage cellular molecules, and it follows that accumulation of such damage, especially to the DNA would be deleterious to the cell. The build-up of DNA damage could, thus, be responsible for the changes in gene expression that we associate with aging.

Numerous damage events

The amount of DNA damage that can occur is considerable. In mice, for example, it is estimated that each cell experiences 40,000 to 150,000 damage events per day. The damage, which happens to nuclear as well as to mitochondrial DNA, can result in apoptosis and/or cellular senescence. DNA repair systems, of course, protect against damage to DNA, but over time, unrepairable damage may accumulate.

Oxidative damage

DNA damage can occur in several ways. Oxidation can damage nucleotides and alter their base-pairing tendencies. Oxidation of guanine by reactive oxygen species, for example, can produce 8-oxo-guanine (Figures 5.52 and 5.53). This oxidized nucleobase commonly produced lesion in DNA arising from action of reactive oxygen species like superoxides. 8-oxoguanine is capable of forming a stable base pairing interaction within a DNA duplex with adenine, potentially giving rise to a mutation when DNA replication proceeds. 8-oxoguanine can be repaired if recognized in time by a DNA glycosylase, which acts to clip out the damaged base and it can then be replaced by the proper one. Polycyclic aromatic hydrocarbons from cigarette smoke, diesel exhaust, or overcooked meat can covalently bind to DNA and, if unrepaired, lead to mutation. Chemical damage to DNA can result in broken or cross-linked DNAs.

Diseases of DNA repair

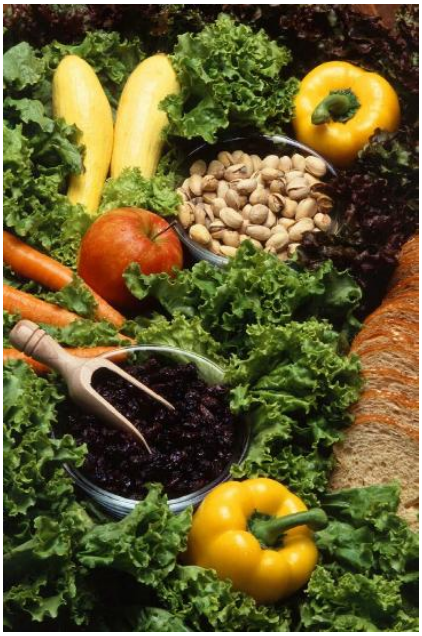
The importance of DNA repair in the aging process is made clear by diseases affecting DNA repair that lead to premature aging. These include Werner syndrome, for whom the life expectancy is 47 years. It arises as a result of loss of two enzymes in base excision repair. People suffering from Cockayne syndrome have a life expectancy of 13 years due to mutations that alter transcription-coupled nucleotide excision repair, which is an important system for fixing oxidative damage.

Further, the life expectancies of 13 species of mammalian organisms correlates with the level of expression of the PARP DNA repair-inducing protein. Interestingly, people who lived past the age of 100 had a higher level of PARP than younger people in the population.

Antioxidants

There is a growing interest in the subject of antioxidants because of health concerns raised by our knowledge of problems created as a result of spontaneous oxidation of biomolecules by Reactive Oxygen Species (ROS), such as superoxide. Antioxidants have the chemical property of protecting against oxidative damage by being readily oxidized themselves, preferentially to other biomolecules.

Biologically, cells have several lines of antioxidant defense. They include molecules, such as vitamins C, A, and E, glutathione, and enzymes that destroy ROS such as superoxide dismutase, catalase, and peroxidases.

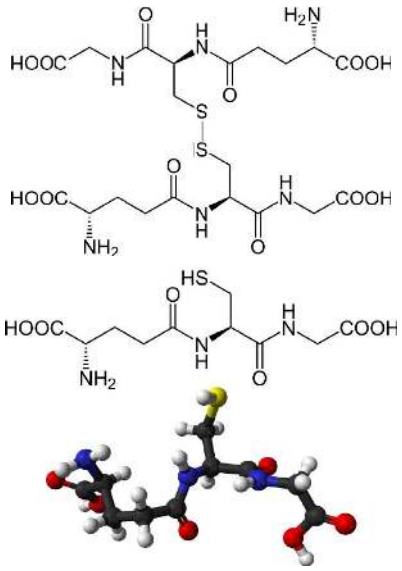


Health effects

Oxidation by ROS is mutagenic and has been linked to atherosclerosis. Nonetheless, randomized studies of oral supplementation of various vitamin combinations have shown no protective effect against cancer and supplementation of Vitamin E and selenium has revealed no decrease in the risk of cardiovascular disease. Further, no reduction in mortality rates as a result of supplementation

with these materials has been found, so the protective effects, if any, of antioxidants on ROS in human health remain poorly understood.

Glutathione



Figures 5.55 & 5.56). The three amino acids in glutathione (glutamate, cysteine, and glycine) are joined in an unusual fashion. The glutamate is joined to the center cysteine by a peptide bond between the R-group carboxyl of glutamate and the α-amine of cysteine. The bond between cysteine and glycine is a normal peptide bond between the α-carboxyl of cysteine and the α-amine of glycine.

The thiol group of cysteine is a reducing agent that reduces disulfide bonds to sulfhydryls in cytoplasmic proteins. This, in turn, is the bridge when two glutathiones get oxidized and form a disulfide bond with each other (Figure 5.56). Glutathione's two oxidative states are abbreviated as follows: GSH (reduced) and GSSG (oxidized).

Disulfide-joined glutathiones can be separated by reduction of their bonds with glutathione reductase, using electrons from NADPH for the reduction.

Non-ribosomal synthesis

Glutathione is not made by ribosomes. Rather, two enzymes catalyze its synthesis. The enzyme γ-glutamylcysteine synthetase catalyzes the joining of the glutamate to the cysteine and then glutathione synthetase catalyzes the peptide bond formation between the cysteine and the glycine. Each step requires energy from ATP.

Essential for life

Glutathione is important for life. Mice lacking the first enzyme involved in its synthesis in the liver die in the first month after birth. In healthy cells, 90% of glutathione is in the GSH state. Higher levels of GSSG correspond to cells that are oxidatively stressed.

Besides reducing disulfide bonds in cells, glutathione is also important for the following:

• Neutralization of free radicals and reactive oxygen species.

• Maintenance of exogenous antioxidants such as vitamins C and E in their reduced forms.

Regulation of the nitric oxide cycle

Resveratrol

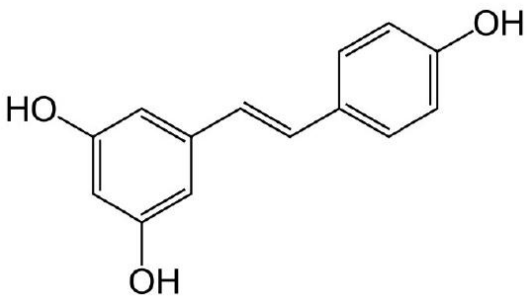


Figure 5.57) is a phenolic compound produced in the skin of plants such as grapes, raspberries, and blueberries, in response to injury or when they are being attacked by pathogens. Numerous health benefits are claimed for the compound, though evidence of such benefits is in short supply. Resveratrol is metabolized rapidly in the body, so it is difficult to maintain levels of it.

Some data indicates resveratrol may improve the functioning of mitochondria. It also acts as an antioxidant and causes concentration of another anti-oxidant, glutathione, to increase. The compound appears to induce expression of manganese superoxide dismutase (protects against reactive oxygen species) and inhibits several phosphodiesterases. This causes an increase in cAMP which results in increases in oxidation of fatty acids, mitochondria formation, gluconeogenesis, and glycogen breakdown. It has been claimed to be the cause of the French Paradox in which drinking of red wine is supposed to give protection for the cardiovascular system. Research data is lacking in support of the claim, however. Resveratrol is known to activate Sirtuin proteins, which play roles in gene inactivation.

Summary

In summary, energy is needed for cells to perform the functions that they must carry out in order to stay alive. At its most basic level, this means fighting a continual battle with entropy, but it is not the only need for energy that cells have.

References

1. Winge, D.R., Mol Cell Biol. 2012 Jul; 32(14): 2647–2652. doi: 10.1128/MCB.00573-12

Energy: Electron Transport & Oxidative Phosphorylation

429

YouTube Lectures

by Kevin

HERE & HERE

YouTube Lectures

by Kevin

HERE & HERE

430

Figure 5.14 - Overview of electron transport (bottom left and top right) and oxidative phosphorylation (top left - yellow box) in the mitochondrion

431

Figure 5.15 - Loss of electrons by NADH to form NAD+. Relevant reactions occur in the top ring of the molecule.

432

Figure 5.16 - Flow of electrons from NADH into the electron transport system. Entry is through complex I

Image by Aleia Kim

Figure 5.17 - Flow of electrons from FADH2 into the electron transport chain. Entry is through complex II.

Image by Aleia Kim

Interactive Learning

Module

HERE

433

Figure 5.18 - Complex I embedded in the inner mitochondrial membrane. The mitochondrial matrix at at the top

Wikipedia

434

Figure 5.19 - Complex II embedded in inner mitochondrial membrane. Matrix is up.

Wikipedia

YouTube Lectures

by Kevin

HERE & HERE

435

Figure 5.20 - Movement of electrons through complex I from NADH to coenzyme Q. The mitochondrial matrix is at the bottom

Image by Aleia Kim

Figure 5.21 - Movement of electrons from succinate through complex II (A->B->C->D->Q). Mitochondrial matrix on bottom.

Image by Aleia Kim

436

Figure 5.22 - Complex II in inner mitochondrial membrane showing electron flow. Matrix is up.

Wikipedia

Figure 5.23 - Coenzyme Q

437

Movie 5.2 - The Q-cycle

Wikipedia

Figure 5.24 - The Q-Cycle Image by Aleia Kim

Figure 5.24 - Complex III

Wikipedia

438

YouTube Lectures

by Kevin

HERE & HERE

Figure 5.25 - The Q-cycle. Matrix is down.

Image by Aleia Kim

439

Figure 5.26 - Movement of electrons and protons through complex IV. Matrix is down

Image by Aleia Kim

Figure 5.25 - Cytochrome c with bound heme Group

Wikipedia

440

Figure 5.27 - Mitochondrial anatomy. Electron transport complexes and ATP synthase are embedded in the inner mitochondrial membrane

Image by Aleia Kim

441

Figure 5.28 - ATP synthase. Protons pass from intermembrane space (top) through the complex and exit in the matrix (bottom).

Image by Aleia Kim

Interactive Learning

Module

HERE

442

Movie 5.3 - ATP Synthase - ADP + Pi (pink) and ATP (red). The view is end-on from the cytoplasmic side viewing the β subunits
Movie 5.3 - ATP Synthase - ADP + Pi (pink) and ATP (red). The view is end-on from the cytoplasmic side viewing the β subunits

443

Figure 5.29 - Important structural features of the ATP synthase

Image by Aleia Kim

444

Figure 5.30 - Loose (L), Tight (T), and Open (O) structures of the F1 head of ATP synthase. Change of structure occurs by rotation of γ-protein (purple) in center as a result of proton movement. Individual α and β units do not rotate

Image by Aleia Kim

445

Figure 5.31 - Respiration overview in eukaryotic cells

Wikipedia

YouTube Lectures

by Kevin

HERE & HERE

446

Rest

ATP High / ADP Low

Oxidative Phosphorylation Low

Electron Transport Low

Oxygen Use Low

NADH High / NAD+ Low

Citric Acid Cycle Slow

Exercise

ATP Low / ADP High

Oxidative Phosphorylation High

Electron Transport High

Oxygen Use High

NADH Low / NAD+ High

Citric Acid Cycle Fast

Interactive Learning

Module

HERE

447

Figure 5.32 - Three inhibitors of electron transport

Image by Aleia Kim

448

Figure 5.33 - Oligomycin A - An inhibitor of ATP synthase

Figure 5.34 - 2,4 DNP - an uncoupler of respiratory control

449

In Cells With Tight Coupling

O2 use depends on metabolism
NAD+ levels vary with exercise
Proton gradient high with no exercise
Catabolism depends on energy needs
ETS runs when OxPhos runs and vice versa

In Cells That Are Uncoupled

O2 use high
NAD+ Levels high
Little or no proton gradient
Catabolism high
OxPhos does not run, but ETS runs rapidly

YouTube Lectures

by Kevin

HERE & HERE

450

451

Figure 5.35 - Alternative oxidase (AOX) of fungi, plants, and protozoa bypasses part of electron transport by taking electrons from CoQ and passing them to oxygen.

452

Figure 5.36 - Structure of an oxygen free radical

Wikipedia

NADPH + 2O2

$NADP^+ + 2O_2^- + H^+$

Figure 5.37 - Three sources of reactive oxygen species (ROS) in cells

Wikipedia

453

454

YouTube Lectures

by Kevin

HERE & HERE

Figure 5.38 A hydroxyl radical

Wikipedia

455

Reduced Glutathione (GSH) + H2O2

Oxidized Glutathione (GSSG) + H2O

Figure 5.40 - Detoxifying reactive oxygen species

Figure 5.39 - Catalase

456

1. O2- + Enzyme-Cu++

O2 + Enzyme-Cu+

2. O2- + Enzyme-Cu+ + 2H+

H2O2 + Enzyme-Cu++

Figure 5.41 - SOD2 of humans

Figure 5.42 3 - Peroxynitrite Ion

Figure 5.44 - SOD1 of humans

Wikipedia

Figure 5.45 - SOD3 of humans

457

Figure 5.43 - Peroxynitrite's effects on cells lead to necrosis or apoptosis

Wikipedia

458

RH + O2 + NADPH + H+

ROH + H2O + NADP+

459

Figure 5.46 - Cytochrome c with its heme group

460

YouTube Lectures

by Kevin

HERE & HERE

Figure 5.47 - Fe2S2 Cluster

Figure 5.48 - Redox reactions for Fe4S4 clusters

461

Figure 5.49 - Tyramine

Figure 5.50 - Phenethylamine

462

Figure 5.51 - Guanine and 8-oxo-guanine

Figure 5.52 - Adenine-8-oxo-guanine base pair. dR = deoxyribose

463

Figure 5.53 - Good antioxidant sources

464

Figure 5.55 - Oxidized glutathiones (GSSG) joined by a disulfide bond

Wikipedia

Figure 5.54 - Structure of reduced glutathione (GSH)

465

Figure 5.56 - Resveratrol

YouTube Lectures

by Kevin

HERE & HERE

466

Graphic images in this book were products of the work of several talented students. Links to their Web pages are below

Click HERE for

Martha Baker’s

Web Page

Click HERE for

Pehr Jacobson’s

Web Page

Click HERE for

Aleia Kim’s

Web Page

Click HERE for

Penelope Irving’s

Web Page

Problem set related to this section HERE

Point by Point summary of this section HERE

To get a certificate for mastering this section of the book, click HERE

Kevin Ahern’s free iTunes U Courses - Basic / Med School / Advanced

Biochemistry Free & Easy (our other book) HERE / Facebook Page

Kevin and Indira’s Guide to Getting into Medical School - iTunes U Course / Book

To see Kevin Ahern's OSU ecampus courses - BB 350 / BB 450 / BB 451

To register for Kevin Ahern's OSU ecampus courses - BB 350 / BB 450 / BB 451

Biochemistry Free For All Facebook Page (please like us)

Kevin Ahern's Web Page / Facebook Page / Taralyn Tan's Web Page

Kevin Ahern's free downloads HERE

OSU's Biochemistry/Biophysics program HERE

OSU's College of Science HERE

Oregon State University HERE

Email Kevin Ahern / Indira Rajagopal / Taralyn Tan

I'm a little mitochondrion
Who gives you energy
I use my proton gradient
To make the ATPs

He's a little mitochondrion
Who gives us energy
He uses proton gradients
To make some ATPs

Electrons flow through Complex II
To traffic cop Co-Q
Whenever they arrive there in
An FADH-two

Electrons flow through Complex II
To traffic cop Co-Q
Whenever they arrive there in
An FADH-two

Tightly coupled is my state
Unless I get a hole
Created in my membrane by
Some di-ni-tro-phe-nol

Yes tightly coupled is his state
Unless he gets a hole
Created in his membrane by
Some di-ni-tro-phenol

Both rotenone and cyanide
Stop my electron flow
And halt the calculation of
My "P" to "O" ratio

Recording by Tim Karplus

Lyrics by Kevin Ahern
Recording by Tim Karplus Lyrics by Kevin Ahern

I'm a Little Mitochondrion

To the tune of "I'm a Lumberjack"

Metabolic Melodies Website HERE

In the catabolic pathways that our cells employ
Oxidations help create the ATP
While they lower Gibbs free energy
Thanks to enthalpy

If a substrate is converted from an alcohol
To an aldehyde or ketone it is clear
Those electrons do not disappear
They just rearrange – very strange

N-A-D is in my ears and in my eyes
Help-ing mol-e-cules get oxidized
Making N-A-D-H then

And the latter is a problem anaerobically
'Cuz accumulations of it muscles hate
They respond by using pyruvate
To produce lactate

Catalyzing is essential for the cells to live
So the enzymes grab their substrates eagerly
If they bind with high affinity
Low Km you see, just as me

N-A-D is in my ears and in my eyes
Help-ing mol-e-cules get oxidized
Making N-A-D-H then

N-A-D

To the tune of "Penny Lane"

Metabolic Melodies Website HERE

Recorded by Tim Karplus

Lyrics by Kevin Ahern
Recorded by Tim Karplus Lyrics by Kevin Ahern

When oxygen's electrons all are in the balanced state

There's twelve of them for oh-two. The molecule is great

But problems sometimes happen on the route to complex IV

Making reactive species that the cell cannot ignore

Oh superoxide dismutase is super catalytic

Keeping cells from getting very peroxynitritic

Faster than a radical, its actions are terrific

Superoxide dismutase is super catalytic

Enzyme, enzyme deep inside

Blocking all the bad oxides

The enzyme's main advantage is it doesn't have to wait

By binding superoxide in a near-transition state

It turns it to an oxygen in mechanism one

Producing "h two oh two" when the cycle is all done

Oh superoxide dismutase you're faster than all them

You've got the highest ratio of kcat over KM

This means that superoxide cannot cause too much mayhem

Superoxide dismutase is faster than all them

Superoxide dismutase

Stopping superoxide's ways

The enzyme's like a ping-pong ball that mechanistic-ly

Bounces between two copper states, plus one and two you see

So S-O-D behaves just like an anti-oxidant

Giving as much protection as a cell could ever want

Oh superoxide dismutase, the cell's in love with you

Because you let electron transport do what it must do

Without accumulation of a radical oh two

Superoxide dismutase - that's why a cell loves you

Superoxide Dismutase

To the tune of "Supercalifragilistiexpialidocious"

Metabolic Melodies Website HERE

Lyrics by Kevin Ahern

No Recording Yet For This Song

This page titled 6.4: Electron Transport and Oxidative Phosphorylation is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **5.2: Electron Transport and Oxidative Phosphorylation** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

6.5: Fats and Fatty Acids

Source: BiochemFFA_6_3.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

In the modern Western world, which is fat and getting fatter, there is a tremendous amount of interest in the metabolism of fat and fatty acids. Fat is the most important energy storage form of animals, storing considerably more energy per carbon than carbohydrates, but its insolubility in water requires the body to package it specially for transport. Surprisingly, fat/fatty acid metabolism is not nearly as tightly regulated as that of carbohydrates. Neither are the metabolic pathways of breakdown and synthesis particularly complicated, either.

Movement of dietary fat

Before we discuss the breakdown and synthesis of fat, let us first discuss the movement of dietary fat and oil (triglycerides - Figure 6.82) in the body. Upon consumption of triglycerides in the diet, they first are solubilized in the digestive system by the churning action of the stomach and the emulsifying properties of the bile acids.

Upon passing into the lumen of the intestine, the triglycerides are acted on first by enzymes known as lipases that use water twice on each triglyceride to release two fatty acids, leaving behind a monoacylglyceride. As shown in Figure 6.83, the fatty acids and the monoacylglyceride are moved across the intestinal wall into the lymph system where they are reassembled back into a triglyceride. In the lymph system triglycerides and other insoluble lipids are packaged into lipoprotein complexes called chylomicrons that enter the blood stream and travel to target cells. The journey of lipids in the body after leaving the digestive system is long and is discussed in more depth HERE.

In the body, fat is stored in specialized cells known as adipocytes. When these cells receive appropriate signals, they begin the breakdown of fat into glycerol and fatty acids.

Breakdown of fat

Breakdown of fat in adipocytes requires catalytic action of three enzymes. The first of these is controlled by binding of hormones to the cell membrane (Figure 6.84). It is the only regulated enzyme of fat breakdown and is known as hormone sensitive triacylglycerol lipase. It removes the first fatty acid from the fat. Diacylglyceride lipase removes the second one and monoacylglyceride lipase removes the third. As noted, only the first one is regulated and it appears to be the rate limiting reaction when active.

Epinephrine activation

As shown in Figure 6.84, activation of hormone sensitive triacylglycerol lipase (HSTL) is accomplished by epinephrine stimulation process and that it overlaps with the same activation that stimulates glycogen breakdown and gluconeogenesis.

This coordination is very important. Each of the pathways stimulated by the epinephrine signaling system aims to provide the body with more materials to catabolize for energy - sugars and fatty acids. The HSTL is inhibited by dephospohrylation and this is stimulated by binding of insulin to its cell membrane receptor.

Perilipin

A protein playing an important roles in regulation of fat breakdown is perilipin. Perilipin associates with fat droplets and helps regulate action of HSTL, the enzyme catalyzing the first reaction in fat catabolism. When perilipin is not phosphorylated, it coats the fat droplet and prevents HSTL from getting access to it. Activation of protein kinase A in the epinephrine cascade, however, results in phosphorylation of both perilipin and HSTL. When this occurs, perilipin loosens its tight binding to the fat droplet, allowing digestion of the fat to begin by HSTL.

Perilipin expression is high in obese organisms and some mutational variants have been associated with obesity in women. Another mutation reduces perilipin expression and is associated with greater lipolysis (fat breakdown) in women. Mice lacking perilipin eat more food than wild-type mice, but gain 1/3 less weight when on the same diet.

Fat synthesis

Synthesis of fat requires action of acyl transferase enzymes, such as glycerol-3 O-phosphate acyl transferase, which catalyzes addition of fatty acids to the glycerol backbone (reaction #1 above). The process requires glycerol-3-phosphate (or DHAP) and three fatty acids. In the first reaction, glycerol-3-phosphate is esterified at position 1 with a fatty acid, followed by a duplicate reaction at position 2 to make phosphatidic acid (diacylglycerol phosphate). This molecule, which is an intermediate in the synthesis of both fats and phosphoglycerides, gets dephosphorylated to form diacylglycerol before the esterification of the third fatty acid to the molecule to make a fat.

Fatty acids released from adipocytes travel in the bloodstream bound to serum albumin. Arriving at target cells, fatty acids are taken up by membrane-associated fatty acid binding proteins, which help control cellular fatty acid uptake by transport proteins. Players in this process include CD36, plasma membrane-associated fatty acid-binding protein, and a family of fatty acid transport proteins (called FATP1-6).

Fatty acid oxidation

Upon arrival inside of target cells, fatty acids are oxidized in a process that chops off two carbons at a time to make acetyl-CoA, which is subsequently oxidized in the citric acid cycle. Depending on the size of the fatty acid, this process (called β-oxidation) will begin in either the mitochondrion (Figure 6.86) or the peroxisomes (see HERE).

Transport

To be oxidized in the mitochondrion, fatty acids must first be attached to coenzyme A (CoA-SH or CoA) and transported through the cytoplasm and the outer mitochondrial membrane. In the mitochondrion's intermembrane space, the CoA on the fatty acid is replaced by a carnitine (Figure 6.87) in order to be moved into the matrix. After this is done, the fatty acid linked to carnitine is transported into the mitochondrial matrix and in the matrix the carnitine is replaced again by coenzyme A. It is in the mitochondrial matrix where the oxidation occurs. The fatty acid linked to CoA (called an acyl-CoA) is the substrate for fatty acid oxidation.

Steps

The process of fatty acid oxidation (Figure 6.88) is fairly simple. The reactions all occur between carbons 2 and 3 (with #1 being the one linked to the CoA) and sequentially include the following steps 1) dehydrogenation to create FADH2 and a fatty acyl group with a double bond between carbons 2 and 3 in the trans configuration; 2) hydration across the double bond to put a hydroxyl group on carbon 3 in the L configuration; 3) oxidation of the hydroxyl group to make a ketone; and 4) thiolytic cleavage to release acetyl-CoA and a fatty acid two carbons shorter than the starting one.

Enzymes of β-oxidation

Two of the enzymes of β-oxidation are notable. The first is acyl-CoA dehydrogenase, which catalyzes the dehydrogenation in the first reaction and yields FADH2. The enzyme comes in three different forms – ones specific for long, medium, or short chain length fatty acids. The first of these is sequestered in the peroxisomes of animals (see below) whereas the ones that work on medium and shorter chain fatty acids are found in the mitochondria. Action of all three enzymes is typically needed to oxidize a fatty acid. Plants and yeast perform β-oxidation exclusively in peroxisomes.

The most interesting of the acyl-CoA dehydrogenases is the one that works on medium length fatty acids. This one, which is the one most commonly deficient in animals, has been associated with sudden infant death syndrome. Reactions two and three in β-oxidation are catalyzed by enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, respectively. The latter reaction yields an NADH.

Thiolase

The second notable enzyme of β-oxidation is thiolase because this enzyme not only catalyzes the formation of acetyl-CoAs in β-oxidation, but also the joining of two acetyl-CoAs (essentially the reversal of the last step of β-oxidation) to form acetoacetyl-CoA – essential for the pathways of ketone body synthesis and cholesterol biosynthesis.

Similarity to citric acid cycle oxidation

It is worth noting that oxidation of fatty acids is chemically very similar to oxidation of the four carbon compounds of the citric acid cycle (Figure 6.89). In fatty acid oxidation, dehydrogenation between carbons 2 and 3 generates electrons which are donated to

FAD to make FADH2 and a trans-bonded intermediate is formed.

The same thing happens in the citric acid cycle reaction catalyzed by succinate dehydrogenase - the trans-bonded molecule is fumarate. Addition of water in the second step of fatty acid oxidation occurs also in the next step of the citric acid cycle catalyzed by fumarase to create malate. Oxidation of the hydroxyl on carbon 3 in β-oxidation is repeated in the citric acid cycle reaction catalyzed by malate dehydrogenase yielding oxaloacetate.

Oxidation of odd chain fatty acids

Though most fatty acids of biological origin have even numbers of carbons, not all of them do. Oxidation of fatty acids with odd numbers of carbons ultimately produces an intermediate with three carbons called propionyl-CoA, which cannot be oxidized further in the β-oxidation pathway.

Metabolism of this intermediate is odd. Sequentially, the following steps occur (Figure 6.90) – 1) carboxylation to make D-methylmalonyl-CoA; 2) isomerization to L-methylmalonyl-CoA; 3) rearrangement to form succinyl-CoA. The last step of the process utilizes the enzyme methylmalonyl-CoA mutase, which uses the B12 coenzyme in its catalytic cycle. Succinyl-CoA can be metabolized in the citric acid cycle.

Peroxisomal oxidation

Long chain fatty acids (typically 22 carbons or more - Figure 6.91) have their oxidation initiated in the peroxisomes, due to the localization of the long acyl-CoA dehydrogenase in that organelle. Peroxisomal fatty acid oxidation is chemically similar to β-oxidation of mitochondria, but there are some differences in the overall process.

Differences

First, since there is no electron transport system in peroxisomes, the reduced electron carriers produced in oxidation there must have their own recycling process. Peroxisomes accomplish this by transferring electrons and protons from FADH2 to O2 to form hydrogen peroxide (H2O2). As a result of this, the lack of electron transport means no proton pump and, consequently, no ATP produced from FADH2 for peroxisomal fatty acid oxidation, making it less efficient that mitochondrial β-oxidation.

Electrons from NADH produced in the third step of the fatty acid oxidation must be shuttled to the cytoplasm and ultimately to the mitochondrion for ATP generation. Peroxisomal oxidation is increased for individuals on a high fat diet. In addition to long chain fatty acids, peroxisomes are also involved in oxidation of branched chain fatty acids, leukotrienes, and some prostaglandins.

Unsaturated fatty acid oxidation

Unsaturated fatty acids complicate the oxidation process a bit (see below), primarily because they have cis bonds, for the most part, if they are of biological origin, and these must be converted to the relevant trans intermediates for β-oxidation. Sometimes the bond must be moved down the chain, as well, in order to be positioned properly. Two enzymes (described below) handle all the necessary isomerizations and moves necessary to oxidize all of the unsaturated fatty acids (Figure 6.92).

Extra enzymes

As noted above, oxidation of unsaturated fatty acids requires two additional enzymes to the complement of enzymes for β-oxidation. If the β-oxidation of the fatty acid produces an intermediate with a cis bond between carbons three and four, cis-Δ3-enoyl-CoA isomerase will convert the bond to a trans bond between carbons two and three and β-oxidation can proceed as normal.

On the other hand, if β-oxidation produces an intermediate with a cis double bond between carbons four and five, the first step of β-oxidation (dehydrogenation between carbons two and three) occurs to produce an intermediate with a trans double bond between carbons two and three and a cis double bond between carbons four and five.

2,4 dienoyl-CoA reductase

The enzyme 2,4 dienoyl CoA reductase reduces this intermediate (using NADPH) to one with a single cis bond between carbons three and four. The newly created cis-bonded molecule is then identical to the one acted on by cis-Δ3-enoyl-CoA isomerase above, which converts it into a regular β-oxidation intermediate, as noted above.

α-oxidation

Yet another consideration for oxidation of fatty acids is α-oxidation. This pathway, which occurs in peroxisomes, is necessary for catabolism of fatty acids that have branches in their chains. For example, breakdown of chlorophyll's phytol group yields phytanic acid (Figure 6.93), which undergoes hydroxylation and oxidation on carbon number two (in contrast to carbon three of β-oxidation), followed by decarboxylation and production of an unbranched intermediate that can be further oxidized by the β-oxidation pathway. Though α-oxidation is a relatively minor metabolic pathway, the inability to perform the reactions of the pathway leads to Refsum's disease where accumulation of phytanic acid leads to neurological damage.

ω-oxidation of fatty acids

In addition to β-oxidation and α-oxidation of fatty acids, which occur in the mitochondria and peroxisomes of eukaryotic cells respectively, another fatty acid oxidation pathway known as ω-oxidation also occurs in the smooth endoplasmic reticulum of liver and kidney cells. It is normally a minor oxidation pathway operating on medium chain fatty acids (10-12 carbons), but gains importance 1) when β-oxidation is not functional or 2) for production of long chain intermediates, such as 20-HETE (20-hydroxyeicosatetraenoic acid), that can function in signaling.

Steps in the process involve 1) oxidation of the terminal methyl group of the fatty acid to an alcohol; 2) oxidation of the alcohol to an aldehyde, and 3) oxidation of the aldehyde group to a carboxylic acid (Figure 6.94). The first oxidation is catalyzed by a mixed function oxidase, and yields 20-HETE if the starting material is arachidonic acid. The last two reactions are catalyzed by alcohol dehydrogenase and each requires NAD^+. After the last oxidation, the fatty acid has carboxyl groups at each end and can be attached to coenzyme A at either end and subsequently oxidized, ultimately yielding succinate.

Regulation of fatty acid oxidation

Breakdown of fatty acids is controlled at different levels. The first is by control of the availability of fatty acids from the breakdown of fat. As noted above, this process is by regulating the activity of hormone-sensitive triacylglycerol lipase (HSTL) activity by epinephrine (stimulates) and insulin (inhibits).

A second level of control of fatty acid availability is by regulation of carnitine acyl transferase (Figure 6.87 - see HERE). This enzyme controls the swapping of CoA on an acyl-CoA molecule for carnitine, a necessary step for the fatty acid to be imported into the mitochondrion for oxidation.

The enzyme is inhibited by malonyl-CoA, an intermediate in fatty acid synthesis. Thus, when fatty acids are being synthesized, import of them into the mitochondrion for oxidation is inhibited. Last, the last enzyme in the β-oxidation cycle, thiolase, is inhibited by acetyl-CoA.

Fatty acid synthesis

Synthesis of fatty acids occurs in the cytoplasm and endoplasmic reticulum of the cell and is chemically similar to the reverse of the β-oxidation process, but with a couple of key differences (Figure 6.95). The first of these occur in preparing substrates for the reactions that grow the fatty acid. Fatty acid synthesis occurs in the cytoplasm of eukaryotic cells. Transport of acetyl-CoA from the mitochondrial matrix occurs when it begins to build up. This happens when the citric acid cycle slows or stops from lack of exercise.

Two molecules can play roles in moving acetyl-CoA to the cytoplasm – citrate and acetylcarnitine. Joining of oxaloacetate with acetyl-CoA in the mitochondrion creates citrate which gets transported across the membrane, followed by action of citrate lyase in the cytoplasm of the cell to release acetyl-CoA and oxaloacetate. Additionally, when free acetyl-CoA accumulates in the mitochondrion, it may combine with carnitine and be transported out to the cytoplasm.

Fatty acid synthase

In animals, six different catalytic activities necessary to fully make palmitoyl-CoA are contained in a single complex called Fatty Acid Synthase. As shown in Figures 6.96 and 6.97, these include 1) transacylases (MAT) for swapping CoA-SH with ACP-SH on acetyl-CoA and malonyl-CoA; 2) a synthase (KS) to catalyze addition of the two carbon unit from the three carbon malonyl-ACP in the first step of the elongation process; 3) a reductase (KR) to reduce the ketone; 4) a dehydrase (DH) to catalyze removal of water; 5) a reductase (ER) to reduce the trans double bond and 6) a thioesterase (TE) to cleave the finished palmitoyl -CoA into palmitic acid and CoA-SH.

In the middle of the complex is a site for binding the ACP portion of the growing fatty acid chain to hold it as the other part of the fatty acid is rotated into positions around the enzyme complex for each catalysis. In bacteria, these six activities are found on separate enzymes and are not part of a complex.

Cytoplasmic reactions

The process of making a fatty acid in the cytoplasm starts with two acetyl-CoA molecules. One is converted to malonyl-CoA by adding a carboxyl group. This reaction is catalyzed by the enzyme acetyl-CoA carboxylase (ACC), the only regulated enzyme of fatty acid synthesis (see below) and the only one separate from the fatty acid synthase. Next, both acetyl-CoA and malonyl-CoA have their CoA portions replaced by a carrier protein known as ACP (acyl-carrier protein) to form acetyl-ACP (catalyzed by acetyl-CoA : ACP transacylase - MAT in Figure 6.97) and malonyl-ACP (catalyzed by malonyl-CoA : ACP transacylase - MAT in Figure 6.97). Joining of a fatty acyl-ACP (in this case, acetyl-ACP) with malonyl-ACP splits out the carboxyl group from malonyl-ACP that was added to it and creates the acetoacyl-ACP intermediate (catalyzed by β-ketoacyl-ACP synthase - KS on Figure 6.97) .

From this point forward, the chemical reactions resemble those of β-oxidation reversed. First, the ketone is reduced to a hydroxyl using NADPH (catalyzed by β-ketoacyl-ACP reductase - KR on Figure 6.97). In contrast to the hydroxylated intermediate of β-oxidation, the intermediate here (D-β- hydroxybutyryl-ACP) is in the D-configuration.

Dehydration

Next, water is removed from carbons 2 and 3 of the hydroxyl intermediate in a reaction catalyzed by 2,3-trans-enoyl-ACP dehydrase - DH on Figure 6.97. This yields a trans doubled-bonded molecule. Last, the double bond is hydrogenated to yield a saturated intermediate by 2,3-trans-enoyl-ACP reductase - ER on Figure 6.97. This completes the first cycle of synthesis.

Additional cycles involve addition of more two-carbon units from malonyl-ACP to the growing chain until ultimately an intermediate with 16 carbons is produced (palmitoyl-ACP). At this point, a thioesterase cleaves the ACP from the palmitoyl-ACP to yield palmitic acid and the cytoplasmic synthesis ceases.

Regulation of fatty acid synthesis

Acetyl-CoA carboxylase, which catalyzes synthesis of malonyl-CoA, is the only regulated enzyme in fatty acid synthesis. Its regulation involves both allosteric control and covalent modification. The enzyme is known to be phosphorylated by both AMP Kinase and Protein Kinase A.

Dephosphorylation is stimulated by phosphatases activated by insulin binding. Dephosphorylation activates the enzyme and favors its assembly into a long polymer, while phosphorylation reverses the process. Citrate acts as an allosteric activator and may also favor polymerization. Palmitoyl-CoA allosterically inactivates it.

Elongation past 16 carbons

Elongation to make fatty acids longer than 16 carbons occurs in the endoplasmic reticulum and is catalyzed by enzymes described as elongases. Mitochondria also can elongate fatty acids, but their starting materials are generally longer than 16 carbons long.

The mechanisms in both environments are similar to those in the cytoplasm (a malonyl group is used to add two carbons, for example), but CoA is attached to the intermediates, not ACP. Further, whereas cytoplasmic synthesis employs the fatty acid synthase complex, the enzymes in these organelles are separable and not part of a complex.

Desaturation of fatty acids

Fatty acids are synthesized in the saturated form and desaturation occurs later - in the endoplasmic reticulum. Reactions to elongate the fatty acid (with elongases) may also occur to make unsaturated fatty acids of varying lengths. Desaturases are named according to the location of the double bonds they introduce in fatty acids. The delta (Δ) system numbers the carbon at the carboxyl end as number 1 and the omega (ω) number system numbers the carbon at the methyl end as number 1 (Figure 6.98). Humans have desaturases named as Δ5, Δ6, and Δ9. A Δ9 desaturase, for example, could convert stearic acid into oleic acid, because stearic acid (see HERE) is a saturated 18 carbon fatty acid and oleic acid is an 18 carbon fatty acid with only one double bond - at position Δ9.

Polyunsaturated fatty acids

Polyunsaturated fatty acids require the action of multiple enzymes and (in some cases) the action of elongases. Arachidonic acid, for example, is a 20 carbon fatty acid with four double bonds and its synthesis requires both an elongase (to increase the length of

the fatty acid from 16 to 20) and multiple desaturases - one for each desaturated double bond.

Animals are limited in the fatty acids they can make, due to an inability of their desaturases to catalyze reactions beyond carbons Δ9. Thus, humans can make oleic acid, but cannot synthesize linoleic acid (Δ9,12) or linolenic acid (Δ9,12,15). Consequently, these two must be provided in the diet and are referred to as essential fatty acids.

Almost all desaturases make cis, not trans double bonds. There are a few minor exceptions to this, in cattle, for example (Figure 6.99). The trans fatty acids found in trans fat of prepared food are produced not by biological processes, but rather by the process of partial hydrogenation of unsaturated fats.

Unusual oxidation reaction

Removal of electrons and protons from a fatty acid to create a double bond is an oxidation reaction and these electrons, must have a destination. The path they take is a bit complex. It involves NAD(P)H, O2, two membrane-bound cytochromes, the membrane bound desaturase, and the fatty acid.

In the electron transfer, the O2 is reduced to two molecules of H2O. This reduction requires four electrons and four protons. Two electrons and two protons come from the fatty acid to form the double bond on it. Two electrons come from the NAD(P)H via the cytochromes and two protons come from the aqueous solution.

Prostaglandin synthesis

The pathway for making prostaglandins and related molecules, such as the leukotrienes, prostacyclin, and thromboxanes is an extension of the synthesis of fatty acids (Figure 6.100).

Prostaglandins, known as eicosanoids because they contain 20 carbons, are synthesized in cells from arachidonic acid whenever it has been cleaved from membrane lipids. Prostaglandins are important for many physiological phenomena in the body, including swelling and pain and reduction of their levels is a strategy of some painkillers, such as aspirin (see below). Inflammation arising from bee stings, for example, occurs because bee (and snake) venom contains mellitin, an activator of PLA2 activity (Figure 6.100). There are two strategies for reducing prostaglandin production and the pain associated with it.

Phospholipase A2

Action of phospholipase enzymes on glycerophospholipids produces fatty acids and either glycerol-3-phosphate or other substances. Figure 6.101 shows cleavage sites on phospholipids that are targeted by different phospholipases. Phospholipase A1 (PLA1), for example, cleaves the fatty acid from position one of the glycerophospholipid and phospholipiase D (PLD) cleaves the R group from the phosphate part of the molecule.

Since the fatty acid on position #2 (where PLA2 cuts) is most commonly unsaturated, PLA2 is an important phosopholipase for hydrolyzing the unsaturated fatty acid known as arachidonic acid from glycerophospholipids. Release of arachidonic acid from membranes is necessary for synthesis of prostaglandins.

Inhibition of the release of arachidonic acid from membranes is the mechanism of action of steroidal anti-inflammatory drugs. They block action of phospholipase A2 (PLA2 - Figure 6.101) which cleaves arachidonic acid from membrane lipids.

Lipocortin

Lipocortin (also called annexin) is a protein that inhibits action of PLA2. Synthesis of lipocortin is stimulated by glucocorticoid hormones, such as cortisol, and is used in some treatments to reduce swelling/inflammation when it is severe and untreatable by non-steroidal drugs.

Second strategy

Synthesis of the prostanoid compounds (prostaglandins, prostacyclin, and thromboxanes) depends on conversion of arachidonic acid to prostaglandins G2 and H2 by COX enzymes. A non-steroidal strategy for decreasing production of prostaglandins then is to inhibit the enzyme that catalyzes their synthesis from arachidonic acid (Figure 6.102). This enzyme is known as prostaglandin synthase, but is more commonly referred to as a cyclooxygenase (or COX) enzyme.

COX enzymes come in at least two forms in humans - COX-1, COX-2. A third form known as COX-3 has been reported as a splice variant of COX-1, but information about it is unclear. COX-1 and COX-2 are very similar in structure (70 kD and 72 kD, respectively, and 65% amino acid sequence homology), but coded by different genes.

COX-1 is synthesized constitutively whereas COX-2 displays inducible expression behavior and has a more specific pattern of tissue expression. COX-2 enzymes are expressed in increasing amounts in areas of growth and inflammation.

Non-steroidal drugs

Molecules inhibiting cyclooxygenases are known as non-steroidal anti-inflammatory drugs (NSAIDs). Molecules in this class include aspirin, ibuprofen, vioxx, and celebrex.

Targeting inhibitors

Some NSAID inhibitors, such as aspirin, bind to all types of COX enzymes. Newer COX inhibitors target the COX-2 enzyme specifically because it was believed to be a better target for relief of joint pain than COX-1 enzymes which are synthesized by most cells. COX-2 enzymes are found more specifically in joints so the thinking was that specific inhibition of them would not affect the COX-1 enzymes which are important for producing prostaglandins that help maintain gastric tissue.

Numerous COX-2 - specific inhibitors were developed - celecoxib, etoricoxib, and rofecoxib (Vioxx), for example. Unfortunately, the COX-2 specific inhibitors are associated with some serious side effects, including a 37% increase in incidence of major cardiovascular events in addition to some of the gastrointestinal problems of NSAIDs.

Imbalance

The increased risk of heart attack, thrombosis, and stroke are apparently due to an imbalance between prostacyclin (reduced by inhibitors) and thromboxanes (not reduced by the inhibitors). Prostacyclin (made from prostaglandin H2 by prostacyclin synthase) is a special prostaglandin that inhibits activation of blood platelets in the blood clotting process and acts as a vasodilator. Thromboxanes counter prostacyclin, causing vasoconstriction and activating blood platelets for clotting. Due to imbalances in these opposite acting molecules resulting from COX-2-specific inhibition, Vioxx, was withdrawn from the market in September, 2004, due to health concerns.

Other compounds known to inhibit COX enzymes include some flavonoids, some components of fish oil, hyperforin, and vitamin D.

Connections to other pathways

There are several connections between fats and fatty acid metabolism and other metabolic pathways. Diacylglycerol (DAG - Figure 6.105), which is produced by removal of a phosphate from phosphatidic acid, is an intermediate in fat synthesis and also a messenger in some signaling systems. Phosphatidic acid, of course, is a branch intermediate in the synthesis of triacylglycerols and other lipids, including phosphoglycerides.

Fatty acids twenty carbons long based on arachidonic acid (also called eicosanoids) are precursors of the leukotrienes, prostaglandins, thromboxanes, and endocannabinoids.

Acetyl-CoA from β-oxidation can be assembled by the enzyme thiolase to make acetoacetyl-CoA, which is a precursor of both ketone bodies and the isoprenoids, a broad category of compounds that include steroid hormones, cholesterol, bile acids, and the fat soluble vitamins. In plants, acetyl-CoA can be made into carbohydrates in net amounts via the glyoxylate cycle.

Fat, obesity, and hunger

Obesity is an increasing problem in the western world. It is, in fact, the leading preventable cause of death worldwide. In 2014, over 600 million adults and 42 million children in the world were classified as obese, a condition when their body mass index is over 30 kg/m2 (Figure 6.106). The body mass index of a person is obtained by dividing a person’s weight by the square of their height. At a simple level, obesity arises from consumption of calories in excess of metabolic need, but there are many molecular factors to consider.

Adipokines

Adipokines are adipose tissue-synthesized cytokines. The class of molecules includes leptin (first discovered adipokine) and hundreds of other such compounds. These include adiponectin (regulates glucose levels and fatty acid oxidation), apelin (control of blood pressure, angiogenesis promotion, vasodilator release, increased water intake), chemerin (stimulation of lipolysis, adipocyte differentiation, link to insulin resistance), and resistin (links to obesity, type II diabetes, LDL production in liver), among others.

Resistin

Resistin is an adipokine peptide hormone with numerous associated negative health effects. Injection of the hormone into mice results in increased resistance to insulin, a phenomenon of type 2 diabetes.

Resistin is linked to increased inflammation and serum levels of it correlate with increased obesity, though direct linkage of it to obesity is controversial. Resistin stimulates production of LDLs in the liver, supporting increased levels in the arteries. Resistin also adversely impacts the effects of statin drugs used to control levels of cholesterol in the body.

Leptin

Leptin is a peptide hormone (adipokine) made in adipose cells that negatively impacts hunger and regulates energy balance. It is countered by ghrelin, also known as the hunger hormone. Both hormones act in the hypothalamus where hunger is controlled. When leptin levels are higher due to higher levels of body fat, hunger is suppressed, but when levels of leptin are lower (less body fat), then appetite increases.

Notably, leptin is also made in places besides adipose tissue and leptin receptors are found in places besides the hypothalamus, so the hormone has other effects in the body. When sensitivity to leptin changes, increased obesity can result. In mice, deletion of leptin function by mutation results in mice with voracious appetites and extreme obesity. Deletion of the leptin receptor gene in mice results in the same phenotype. Eight humans with leptin mutations all suffer from extreme obesity in infancy.

Physiology

Leptin is produced primarily by cells in white adipose tissue, but is also made in brown adipose tissue, ovaries, skeletal muscle, stomach, mammary epithelial cells and bone marrow.

Leptin levels

Leptin levels in the body are highest between midnight and early morning, presumably to suppress appetite. Though it is produced by fat cells, levels of leptin in humans do not strictly reflect levels of fat. For example, early in fasting, leptin levels fall before fat levels fall. Sleep deprivation can reduce leptin levels, as can increasing levels of testosterone and physical exercise.

Increasing estrogen, however, increases leptin levels. Emotional stress and insulin can increase leptin levels. Obesity increases leptin levels, but doesn’t fully suppress appetite. Leptin resistance in these individuals is an important consideration, lessening the effects of the hormone on appetite.

Blocking leptin action

In the medial hypothalamus, leptin stimulates satiety and in the lateral hypothalamus, leptin inhibits hunger. Lesions in the lateral hypothalamus that block the ability to sense hunger result in anorexia (there are other causes of anorexia, though) and lesions in the medial hypothalamus cause excess hunger (no satiety). Neuropeptide Y is a potent hunger promoter whose receptors in the arcuate nucleus can be bound and blocked by leptin. Leptin levels are more sensitive to decreasing food intake than increasing food intake meaning that in humans the hormone plays a bigger role with respect to appetite than to levels of fat in the body.

At the molecular level, binding of leptin to the Ob-Rb receptor causes down-regulation of synthesis of endocannabinoids, whose normal function is to increase hunger. High fructose diets have been associated with reduced levels of leptin and of leptin receptor.

Ghrelin

Ghrelin is a peptide hormone made by cells in the gastrointestinal tract when the stomach is empty. Stretching of the stomach reduces the expression of the hormone. Ghrelin exerts its effects on the central nervous system to increase appetite and it is an unusual peptide in being able to cross the blood-brain barrier. The ghrelin receptor in the brain is found on the same cells as the leptin receptor (arcuate nucleus). Leptin can counter the ghrelin effect by decreasing hunger.

Behavioral effects

Activation of ghrelin occurs after processing the zymogen form of the hormone (pre-proghrelin) followed by linkage of an octanoic acid to a serine at position 3. Circulating levels of ghrelin increase before eating and decrease afterwards. There appears to be a dose dependence for ghrelin on the amount of food consumed. Ghrelin increases food seeking behavior and there is a negative correlation between levels of ghrelin and weight.

Neuropeptide Y

Neuropeptide Y is a neuropeptide neurotransmitter produced by neurons of the sympathetic nervous system. It acts as a vasoconstrictor and favors growth of fat tissue. It appears to stimulate food intake, fat storage, relieve anxiety/stress, reduce pain perception, and lower blood pressure. Blockage of neuropeptide Y receptors in the brain of rats decreases food intake.

Stress effects

In mice and monkeys, repeated stress and high fat, high sugar diets stimulate neuropeptide Y levels and cause abdominal fat to increase.

High levels of neuropeptide Y may also help individuals to recover from post-traumatic stress disorder and to reduce the fear response. It may also protect against alcoholism. Mice lacking the ability to make neuropeptide Y have a higher voluntary consumption of alcohol and are less sensitive to its effects. The neuropeptide Y receptor is a G-protein-coupled receptor in the 7-transmembrane domain family.

Metabolism: Fats and Fatty Acids

564

YouTube Lectures

by Kevin

HERE & HERE

565

Figure 6.83 - Movement of dietary triglycerides

Image by Aleia Kim

Figure 6.82 - Trimyristin - A triacylglyceride

566

Figure 6.84 - Breakdown of fat in adipocytes

Image by Pehr Jacobson

567

Figure 6.85 - Synthesis of fat from phosphatidic acid (phosphatidate)

Image by Penelope Irving

Synthesis of Phosphatidic Acid from Glycerol-3-phosphate

1. Glycerol-3-phosphate + Acyl-CoA <=> Monoacylglycerol phosphate + CoA-SH

2. Monoacylglycerol phosphate + Acyl-CoA <=> Phosphatidic acid + CoA-SH

568

Figure 6.86 - Mitochondria - site of β-oxidation

Figure 6.87 - Transport of fatty acid (acyl group) across mitochondrial inner membrane

Image by Aleia Kim

569

Figure 6.88 - Four reactions in β-oxidation

Image by Aleia Kim

YouTube Lectures

by Kevin

HERE & HERE

570

Figure 6.89 - Similar reactions for fatty acid oxidation and oxidation of 4-carbon compounds in the citric acid cycle

Image by Aleia Kim

571

Figure 6.90 - Metabolism of propionyl-CoA

Image by Pehr Jacobson

572

In beta oxidation, it just occurred to me

The process all takes place ‘tween carbons two and three

Some hydrogens are first removed to FADH2

Then water adds across the bond, the H to carbon two

Hydroxyl oxidation’s next, a ketone carbon three

Then thiolase catalysis dissects the last two C’s

The products of the path, of course, are acetyl-CoAs

Unless there were odd carbons, hence propionyl-CoA

Figure 6.91 - Cerotic acid - A long chain fatty acid with 26 carbons

573

Figure 6.92 - Unsaturated fatty acid oxidation

YouTube Lectures

by Kevin

HERE & HERE

574

Figure 6.93 - Phytanic acid

575

Figure 6.94 - ω Oxidation

Wikipedia

576

Figure 6.95 - Fatty acid synthesis is the reverse of fatty acid oxidation chemically

577

Figure 6.96 - One round of fatty acid synthesis

image by Aleia Kim

Figure 6.97 - Fatty acid synthase complex

578

579

For fatty acid synthesis, I must reverse the path

Of breaking fatty acids, though you’ll wonder ‘bout the math

Each cycle of addition starts with carbons one two three

Yet products of reactions number carbons evenly

The reason is that CO2 plays peek-a-boo like games

By linking to an Ac-CoA then popping off again

Reactions are like oxidations ‘cept they’re backwards here

Reduction, dehydration, then two hydrogens appear

The product of the process is a 16 carbon chain

The bonds are saturated. No double ones remain

For them desaturases toil to put in links of cis

In animals to delta nine, but no more go past this

And last there’s making longer ones eicosanoidic fun

They’re made by elongases in the e. reticulum

Kevin Ahern

YouTube Lectures

by Kevin

HERE & HERE

Figure 6.98 - Carbon numbering schemes for fatty acids

Image by Pehr Jacobson

580

Stearoyl-CoA + 2 Cytochrome b5 (red) + O2 + 3 H+ + NADPH

Oleoyl-CoA + 2 Ferricytochrome b5 (ox) + 2 H2O + NADP+

Desaturase Reaction to Oxidize Stearic Acid

Figure 6.99 - Elaidic acid - A rare trans fatty acid in biology

581

Figure 6.100 - Eicosanoid synthesis pathways

Image by Pehr Jacobson

582

583

Figure 6.101 - Cleavage sites for four phospholipiases on a glycerophospholipid - phospholipases A1 (PLA1), A2 (PLA2), C (PLC), and D (PLD)

YouTube Lectures

by Kevin

HERE & HERE

584

Figure 6.102 - Catalytic activity of cyclooxygenase and peroxidase in making prostaglandins

Image by Pehr Jacobson

585

Figure 6.103 - Synthesis of prostaglandins from prostaglandin H2 (red)

Image by Pehr Jacobson

Figure 6.104 - Two NSAIDs

586

Figure 6.105 - Diacylglycerol

Figure 6.106 - Obesity worldwide - females (top) and males (bottom)

Wikipedia

587

Figure 6.107 Leptin

Wikipedia

588

YouTube Lectures

by Kevin

HERE & HERE

Figure 6.108 Neuropeptide Y

589

590

Figure 6.109 - Pre-proghrelin

591

Graphic images in this book were products of the work of several talented students. Links to their Web pages are below

Click HERE for

Martha Baker's

Web Page

Click HERE for

Pehr Jacobson's

Web Page

Click HERE for

Aleia Kim's

Web Page

Click HERE for

Penelope Irving's

Web Page

Problem set related to this section HERE

Point by Point summary of this section HERE

To get a certificate for mastering this section of the book, click HERE

Kevin Ahern's free iTunes U Courses - Basic / Med School / Advanced

Biochemistry Free & Easy (our other book) HERE / Facebook Page

Kevin and Indira's Guide to Getting into Medical School - iTunes U Course / Book

To see Kevin Ahern's OSU ecampus courses - BB 350 / BB 450 / BB 451

To register for Kevin Ahern's OSU ecampus courses - BB 350 / BB 450 / BB 451

Biochemistry Free For All Facebook Page (please like us)

Kevin Ahern's Web Page / Facebook Page / Taralyn Tan's Web Page

Kevin Ahern's free downloads HERE

OSU's Biochemistry/Biophysics program HERE

OSU's College of Science HERE

Oregon State University HERE

Email Kevin Ahern / Indira Rajagopal / Taralyn Tan

592

When Acids Get Oxidized

To the tune of "When Johnny Comes Marching Home"

Metabolic Melodies Website HERE

The fatty acids carried by
CoA, CoA
Are oxidized inside the
mi-to-chon-dri-ay

They get to there as you have seen
By hitching rides on carnitine
Then it goes away
When acids get oxidized

Electrons move through membranes, yes
It's true, it's true
They jump from complex I onto
Co-Q, Co-Q

The action can be quite intense
When building proton gradients
And its good for you
When acids get oxidized

The protons pass through complex V
You see, you see
They do this to make lots of
A-TP, TP

The mechanism you should know
Goes through the stages L-T-O
So there's energy
When acids get oxidized

Recording by Tim Karplus

Lyrics by Kevin Ahern
Recording by Tim Karplus Lyrics by Kevin Ahern

593

When Acids Are Synthesized

To the tune of “When Johnny Comes Marching Home”

Metabolic Melodies Website HERE

The 16 carbon fatty acid, palmitate
Gets all the carbons that it needs from acetate
Which citric acid helps release
From mitochondri - matrices
Oh a shuttle's great
When acids are synthesized

Carboxylase takes substrate and it puts within
Dioxy carbon carried on a biotin
CoA's all gain a quick release
Replaced by larger ACPs
And it all begins
When acids are synthesized

A malonate contributes to the growing chain
Two carbons seven times around again, again
For saturated acyl-ates
There's lots of N-A-DPH
That you must obtain
When acids are synthesized

Palmitic acid made this way all gets released
Desaturases act to make omega-threes
The finished products big and small
Form esters with a glycerol
So you get obese
When acids are synthesized

Recording by Tim Karplus

Lyrics by Kevin Ahern
Recording by Tim Karplus Lyrics by Kevin Ahern

This page titled 6.5: Fats and Fatty Acids is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **6.3: Fats and Fatty Acids** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

6.6: Amino Acids and the Urea Cycle

Source: BiochemFFA_6_5.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

In contrast to some of the metabolic pathways described to this point, amino acid metabolism is not a single pathway. The 20 amino acids have some parts of their metabolism that overlap with each other, but others are very different from the rest. In discussing amino acid metabolism, we will group metabolic pathways according to common metabolic features they possess (where possible). First, we shall consider the anabolic pathways.

Transamination

Before beginning discussion of the pathways, it is worthwhile to discuss a reaction common to the metabolism of most of the amino acids and other nitrogen-containing compounds and that is transamination. In cells, nitrogen is a nutrient that moves from one molecule to another in a sort of hand-off process. A common transamination reaction is shown on the next page.

A specific reaction of this type is shown in Figure 6.134.

Glutamate and glutamine play central roles in transamination, each containing one more amine group than α-ketoglutarate and glutamate, respectively. Transamination reactions, as noted earlier, occur by a ping-pong mechanism and involve swaps of amines and oxygens in Schiff base reactions. Two amino acids, glutamine and asparagine are the products of gaining an amine in their respective R-groups in reactions involving ammonium ion.

Synthesis varies

It is also important to recognize that organisms differ considerably in the amino acids that they can synthesize. Humans, for example, cannot make 9 of the 20 amino acids needed to make proteins, and the number of these that can be synthesized in needed amounts varies between adults and children.

Amino acids that cannot be made by an organism must be in the diet and are called essential amino acids. Non-essential amino acids are those an organism can make in sufficient quantities (Figure 6.135). Though amino acids do not have a common pathway of metabolism, they are often organized in “families” of amino acids with overlapping metabolic reactions common to members of each group. To designate amino acid families in the text we will use a blue font for headings to distinguish them.

α-ketoglutarate family

This family of amino acids arises from α-ketoglutarate of the citric acid cycle. It includes the amino acids glutamic acid, glutamine, proline, and arginine. It is also called the glutamate family, since all the amino acids in it derive from glutamate.

Glutamate

α-ketoglutarate is readily converted to glutamate in transamination reactions, as noted above. It can also be produced by the enzyme glutamate dehydrogenase, which catalyzes the reaction below (in reverse) to make glutamate.

In the forward direction, the reaction is a source of ammonium ion, which is important both for the urea cycle and for glutamine metabolism. Because it is a byproduct of a citric acid cycle intermediate, glutamate can therefore trace its roots to any of the intermediates of the cycle. Citrate and isocitrate, for example, can be thought of as precursors of glutamate. In addition, glutamate can be made by transamination from α-ketoglutarate in numerous transamination reactions involving other amino acids.

Glutamine

Synthesis of glutamine proceeds from glutamate via catalysis of the enzyme glutamine synthetase, one of the most important regulatory enzymes in all of amino acid metabolism (Figure 6.136).

Regulation of the enzyme is complex, with many allosteric effectors. It can also be controlled by covalent modification by adenylylation of a tyrosine residue in the enzyme (Figure 6.137). In the figure, PA and PD are regulatory proteins facilitating conversion of the enzyme.

Ammonia used in the reaction catalyzed by glutamate synthetase commonly arises from nitrite reduction, amino acid breakdown, or photorespiration. Because it builds ammonia into an amino acid, glutamine synthetase helps reduce the concentration of toxic

ammonia - an important consideration in brain tissue. Some inhibitors of glutamine synthetase are, in fact, the products of glutamine metabolism. They include histidine, tryptophan, carbamoyl phosphate, glucosamine-6-phosphate, CTP, and AMP. The glutamate substrate site is a target for the inhibitors alanine, glycine, and serine. The ATP substrate site is a target for the inhibitors GDP, AMP, and ADP. Complete inhibition of the enzyme is observed when all of the substrate sites of the multi-subunit enzyme are bound by inhibitors. Lower levels of inhibitors results in partial or full activity, depending on the actual amounts.

Proline

Synthesis of proline starts with several reactions acting on glutamate. They are shown below in the green text box.

The L-glutamate-5-semialdehyde, so produced, is a branch point for synthesis of proline or ornithine. In the path to make proline, spontaneous cyclization results in formation of 1-pyrroline-5-carboxylic acid (Figure 6.138).

This, in turn, is reduced to form proline by pyrroline-5-carboxylate reductase.

Arginine

Arginine is a molecule synthesized in the urea cycle and, thus, all urea cycle molecules can be considered as precursors. Starting with citrulline, synthesis of arginine can proceed as shown on the next page. The urea cycle can be seen HERE.

An alternate biosynthetic pathway for making arginine from citrulline involves reversing the reaction catalyzed by nitric oxide synthase. It catalyzes an unusual five electron reduction reaction that proceeds in the following manner

Yet another way to synthesize arginine biologically is by reversal of the arginase reaction of the urea cycle

Arginine can also be made starting with glutamate. This 5 step pathway leading to ornithing is illustrated at the top of the next page (enzymes in blue). Ornithine, as noted above can readily be converted to arginine.

The last means of making arginine is by reversing the methylation of asymmetric dimethylarginine (ADMA - Figure 6.140). ADMA is a metabolic byproduct of protein modification. It interferes with production of nitric oxide and may play a role in cardiovascular disease, diabetes mellitus, erectile dysfunction, and kidney disease.

Serine family

Serine is a non-essential amino acid synthesized from several sources. One starting point is the glycolysis intermediate, 3-phosphoglycerate, (3-PG) in a reaction catalyzed by 3-PG dehydrogenase.

Transamination by phosphoserine aminotransferase produces O-phosphoserine. The phosphate is then removed by phosphoserine phosphatase, to make serine. These reactions are shown below. Phosphoserine phosphatase is missing in the genetic disease known as Williams-Beuren syndrome.

Serine can also be derived from glycine and vice versa. Their metabolic paths are intertwined as will be seen below. Serine is important for metabolism of purines and pyrimidines, and is the precursor for glycine, cysteine, and tryptophan in bacteria, as well as for sphingolipids and folate. Serine in the active site of serine proteases is essential for catalysis. A serine in the active site of acetylcholinesterases is the target of nerve gases and insecticides.

Covalent modification target

Serine in proteins can be the target of glycosylation or phosphorylation. D-serine is the second D-amino acid known to function in humans. It serves as a neuromodulator for NMDA receptors, by serving as a co-agonist, together with glutamate. D-serine is being studied as a schizophrenia treatment in rodents and as a possible biomarker for Alzheimers.

Glycine

As noted, glycine’s metabolism is intertwined with that of serine. This is apparent in the reaction catalyzed by serine hydroxymethyltransferase.

Notably, the previous reaction is also needed for recycling of folate molecules, which are important for single carbon reactions in nucleotide synthesis.

Vertebrates can also synthesize glycine in their livers using the enzyme glycine synthase.

Glycine is a very abundant component of collagen. It is used in the synthesis of purine nucleotides and porphyrins. It is an inhibitory neurotransmitter and is a co-agonist of NMDA receptors with glutamate. Glycine was detected in material from Comet Wild 2.

Cysteine

Cysteine can be synthesized from several sources. One source is the metabolism of the other sulfur-containing amino acid, methionine. This begins with formation of S-Adenosyl-Methionine (SAM), catalyzed by methionine adenosyltransferase.

SAM is a methyl donor for methyl transfer reactions and that is the next step in the pathway - donation of a methyl group (catalyzed by transmethylase)

SAH (S-Adenosylhomocysteine) is cleaved by S-adenosylhomocysteine hydrolase,

Homocysteine can be recycled back to methionine by action of methionine synthase

On the path to making cysteine, homocysteine reacts as follows (catalyzed by cystathionine β-synthase).

Last, cystathionase catalyzes release of cysteine

β-ketobutyrate can be metabolized to propionyl-CoA and then to succinyl-CoA to be used ultimately in the citric acid cycle.

Another route to making cysteine is a two-step process that begins with serine, catalyzed first by serine-O-acetyltransferase

and then by cysteine synthase

Cysteine can be also released from cystine by cystine reductase

Finally, cysteine can be made from cysteic acid by action of cysteine lyase

Aspartate family

Metabolism of aspartic acid is similar to that of glutamate. Aspartic acid can arise from transamination of a citric acid cycle intermediate (oxaloacetate).

Aspartate can also be generated from asparagine by the enzyme asparaginase.

Further, aspartate can be produced by reversal of a reaction in the urea cycle (see HERE)

Aspartate is also a precursor to four amino acids that are essential in humans. They are methionine, isoleucine, threonine, and lysine. Because oxaloacetate can be produced from aspartate, aspartate is an important intermediate for gluconeogenesis when proteins are the energy source.

Asparagine

Asparagine, too, is an amino acid produced in a simple transamination reaction. In this case, the precursor is aspartate and the amine donor is glutamine (catalyzed by asparagine synthetase)

Methionine

Metabolism of methionine overlaps with metabolism of the other sulfur-containing amino acid, cysteine. Methionine is not made in humans (essential) so the pathway shown in Figure 6.141 is from bacteria.

The process begins with phosphorylation of aspartate. Numbers for each catalytic step in the figure are for the enzymes that follow:

1 - Aspartokinase
2 - Aspartate-semialdehyde dehydrogenase
3 - Homoserine dehydrogenase
4 - Homoserine O-transsuccinylase
5 - Cystathionine-γ-synthase
6 - Cystathionine-β-lyase
7 - Methionine synthase

Though humans cannot make methionine by the pathway shown in the figure, they can recycle methionine from homocysteine (a product of S-adenosylmethionine metabolism). This reaction requires the enzyme methionine synthase and Vitamin B12 as a co-

factor.

An alternative pathway of converting homocysteine to methionine involves a prominent liver enzyme, betaine-homocysteine methyltransferase. This enzyme catalyzes the reaction below.

In this reaction, a methyl group is transferred to homocysteine from glycine betaine to make the methionine. Glycine betaine is a trimethylated amine of glycine found in plants. It is a byproduct of choline metabolism.

Bacteria, mitochondria, and chloroplasts use a modified form of methionine, N-formyl-methionine (Figure 6.142), as the first amino acid incorporated into their proteins. Formylation of methionine occurs only after methionine has been attached to its tRNA for translation. Addition of the formyl group is catalyzed by the enzyme methionyl-tRNA formyltransferase

Threonine

Though threonine is chemically similar to serine, the metabolic pathway leading to threonine does not overlap with that of serine. As seen in the figure, aspartate is a starting point for synthesis. Two phosphorylations/dephosphorylations and two reductions with electrons from NADPH result in production of threonine.

Enzymes in Figure 6.143 are as follows:

1 Aspartokinase
2 β-aspartate semialdehyde dehydrogenase
3 Homoserine dehydrogenase
4 Homoserine kinase
5 Threonine synthase

Breakdown of threonine produces acetyl-CoA and glycine. It can also produce α-ketobutyrate, which can be converted to succinyl-CoA for oxidation in the citric acid cycle.

Lysine

To get from aspartate to lysine, nine reactions and two non-enzymatic steps are involved, as seen in Figure 6.144. Enzymes involved in lysine biosynthesis include (numbers correspond to numbered reactions in Figure 6.144):

1 - Aspartokinase
2 - Aspartate-semialdehyde dehydrogenase
3 - 4-hydroxy-tetrahydrodipicolinate synthase
4 - 4-hydroxy-tetrahydrodipicolinate reductase
5 - 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase
6 - Succinyl-diaminopimelate transaminase
7 - Succinyl-diaminopimelate desuccinylase
8 - Diaminopimelate epimerase
9 - Diaminopimelate decarboxylase

Low in cereal grains

Lysine is the essential amino acid found in the smallest quantity in cereal grains, but is found abundantly in legumes. Besides its synthesis and breakdown, lysine can be methylated, acetylated, hydroxylated, ubiquitinated, sumoylated, neddylated, biotinylated, pupylated, and carboxylated within proteins containing it. Hydroxylation of lysine is important for strengthening collagen and acetylation/methylation of lysine in histone proteins play roles in control of gene expression and epigenetics. Besides being used to make proteins, lysine is important for calcium absorption, recovery from injuries, and for production of hormones.

Oral lysine has been used as a treatment for herpes infections (cold sores) but its efficacy is not established and it is not clear by what mechanism is would reduce the duration of the infection or reduce the number of outbreaks of viral infection..

Aromatic amino acids

The aromatic amino acids, tryptophan, phenylalanine, and tyrosine can all be made starting with two simple molecules - PEP and erythrose-4-phosphate (Figure 6.145). All three aromatic amino acids are also important sources of hormones, neurotransmitters,

and even the skin pigment melanin.

Tryptophan synthesis

The proteogenic amino acid with the largest R-group, tryptophan is an essential amino acid distinguished structurally by its indole group. The amino acid is made in bacteria and plants from shikimic acid or anthranilate and serine is used in its synthesis.

Erythrose-4-phosphate and phosphenolpyruvate (PEP) also serve as building blocks of tryptophan. The pathway of its synthesis is shown in Figures 6.146 to 6.148.

Erythrose-4-phosphate and phosphoenolpyruvate (PEP) are joined and then, after one hydrolysis, one dehydration, one oxidation and one reduction, the product is shikimic acid (Figure 6.147).

Shikimic acid is converted to chorismic acid in three steps, as shown in Figure 6.147. Finally, synthesis of tryptophan from chorismic acid is shown in Figure 6.148.

Regulation

Regulation of tryptophan synthesis in bacteria occurs partly via a process called attenuation that operates through the trp operon. In this mechanism, low levels of tryptophan slow ribosomal movement (and translation) through the operon. This is particularly important because bacteria can have transcription and translation occurring simultaneously. Slowing translation due to low tryptophan levels allows a transcription termination mechanism to be inhibited. Since translation only slows when tryptophan is in short supply, premature termination of transcription occurs when tryptophan is abundant (see also HERE).

Besides its importance for making proteins, tryptophan is an important precursor of serotonin (neurotransmitter), melatonin (hormone), niacin (vitamin), and auxin (plant hormone). The two pathways leading from tryptophan to three of these molecules is shown in Figure 6.149.

Melatonin

Melatonin is a compound made from tryptophan that is found in a wide spectrum of biological systems, including plants, animals, fungi, and bacteria. In animals, it acts as a hormone for circadian rhythm synchronization, signaling the onset of darkness each day. It has effects on the timing of sleep, seasonal effects, and can affect blood pressure, among other physiological phenomena. It can cross cell membranes, as well as the blood-brain barrier. Melatonin is a potent anti-oxidant and provides protective functions for nucleic acids. It is used sometimes to help in treatment of sleep disorders. Some reports have indicated that children with autism have abnormal melatonin pathways with low levels of the hormone.

Blue light

Melatonin production is affected by blue light and may be linked to sleep abnormalities for people using computer monitors after dark. To protect against this, some computer programs are available that reduce the screen's blue light output in the evenings. Special eyeglasses that block blue light are also available. Though melatonin is linked to sleep in some animals (including humans), nocturnal animals are activated by increasing melatonin levels. Varying day/night lengths during the year alter melatonin production and provide biological signals of the seasons. These are especially important in the seasonal coloring and breeding habits of some animals. Melatonin is present in cherries, bananas, grapes, rice, cereals, olive oil, wine, and beer.

Serotonin

Serotonin, or 5-hydroxytryptamine, is a monoamine neurotransmitter derived from tryptophan. Blood platelets store serotonin and release it when they bind to a clot, causing vasoconstriction. Serotonin plays a role in cognitive functions and enhances memory and learning. Serotonin is widely thought to be a contributor to feelings of happiness and well-being. Some common anti-depressant drugs, including Prozac, Paxil and Zoloft, act to modulate action of serotonin at synapses.

Niacin

Niacin is also known as Vitamin B3 and nicotinic acid. Niacin can be made from tryptophan and people who have the inability to absorb tryptophan in the digestive system exhibit symptoms similar to niacin deficiency.

Extreme deficiency of niacin in the diet leads to the disease known as pellagra, while insufficient amounts of niacin in the diet are linked with nausea, anemia, headaches, and tiredness. A diet that is primarily composed of grains like corn can lead to niacin

deficiency, because the niacin in these sources is not readily bioavailable. Treatment of the grain with alkali, as in the traditional Mexican practice of soaking corn in lime, can make the niacin more easily absorbed from food.

Niacin is related to pyridine and the amide form of it is nicotinamide, an important component of NAD+/NADH and NADP+/NADPH. The last pairs of molecules are essential as electron acceptors/carriers for most cellular oxidation-reduction reactions.

Auxins

Auxins are plant growth hormones derived from tryptophan. The most important of these is indole-3-acetic acid (Figure 6.151). Auxins are involved in almost every aspect of plant growth and development. They activate proteins, such as expansins and various enzymes that modify the structure of cell wall components, to loosen the cell walls of a plant and stimulate elongation of cells. In the presence of cytokinins, auxins stimulate cell division. Auxins are also involved in the maintenance of meristems and in cell patterning and organogenesis. Auxins are crucial for establishing root primordia as well as for elongation of root hairs. Auxins play important roles in organizing the xylem and phloem of plants, and it has long been known that plant callus tissue can be made to differentiate into shoots or roots, depending on the relative concentrations of auxins and cytokinins supplied in the medium.

Agrobacterium tumefaciens, a bacterium which infects a wide variety of plants, inserts its own DNA, including genes necessary for the synthesis of plant hormones, into its host's cells. The subsequent overproduction of auxins stimulates the growth of tumors (called crown galls) on the plant (Figure 6.153).

Phenylalanine

Phenylalanine is an essential, hydrophobic amino acid in humans that is a precursor of tyrosine and since tyrosine is a precursor of several important catecholamines, phenylalanine is, thus, a precursor of them as well.

PKU

Phenylalanine is linked to the genetic disease phenylketonuria (PKU) which arises from an inability to metabolize the amino acid in people lacking (or deficient in) the enzyme phenylalanine hydroxylase. If left untreated, the disease can cause brain damage and even death, but if detected early, it can be easily managed by carefully monitoring dietary intake of the amino acid. Because of this, newborns are routinely tested for PKU. Phenylalanine is a component of the artificial sweetener known as aspartame (Nutrasweet - Figure 6.154) and is consequently dangerous for people suffering from this disorder.

Biosynthesis of phenylalanine in bacteria overlaps with synthesis of tryptophan. The branch occurs at chorismic acid where the enzyme chorismate mutase catalyzes a molecular rearrangement to produce prephenate.

Proton attack on prephenate results in loss of water and carbon dioxide to yield phenylpyruvate.

Transamination of phenylpyruvate yields phenylalanine.

Alternatively, phenylalanine can obtain its amine group in a transamination reaction from alanine.

Hydroxylation of phenylalanine by aromatic amino acid hydroxylase (phenylalanine hydroxylase) yields tyrosine.

Tyrosine

Because tyrosine is made from phenylalanine and the latter is an essential amino acid in humans, it is not clear whether to classify tyrosine as essential or non-essential. Some define it as a conditionally essential amino acid. Others simply categorize it as non-essential.

As noted above, tyrosine can arise as a result of hydroxylation of phenylalanine. In addition, plants can synthesize tyrosine by oxidation of prephenate followed by transamination of the resulting 4-hydroxyphenylpyruvate (Figure 6.155).

The hydroxyl group on tyrosine is a target for phosphorylation by protein kinase enzymes involved in signal transduction pathways (Figure 6.156). When located in membranes, these enzymes are referred to as receptor tyrosine kinases and they play important roles in controlling cellular behavior/response.

In photosystem II of chloroplasts, tyrosine, at the heart of the system, acts as an electron donor to reduce oxidized chlorophyll. The hydrogen from the hydroxyl group of tyrosine is lost in the process, requiring re-reduction by four core manganese clusters.

Tyrosine is also important in the small subunit of class I ribonucleotide reductases where it forms a stable radical in the catalytic action of the enzyme (see HERE).

Tyrosine metabolites

Tyrosine is a precursor of catecholamines, such as L-dopa, dopamine, norepinephrine, and epinephrine (Figure 6.157). The thyroid hormones triiodothyronine (T3) and thyroxine (T4) are also synthesized from tyrosine. As shown in Figure 6.158, this involves a series of iodinations of tyrosines side-chains of a protein known as thyroglobulin. Combinations of iodinated tyrosines give rise to thyroxine and triiodothyronine. These are subsequently cleaved from the protein and released into the bloodstream.

Oxidation and polymerization of tyrosine is involved in synthesis of the family of melanin pigments. Tyrosine is involved in the synthesis of at least two types - eumelanin and pheomelanin (Figure 6.159).

Another molecule derived from tyrosine is the benzoquinone portion of Coenzyme Q (CoQ). This pathway requires the enzyme HMG-CoA Reductase and since this enzyme is inhibited by cholesterol-lowering statin drugs, CoQ can be limited in people being treated for high cholesterol levels.

Dopamine

Dopamine plays several important roles in the brain and body. A member of the catecholamine and phenethylamine families, its name comes from the fact that it is an amine made by removing a carboxyl group from L-DOPA. Dopamine is synthesized in the brain and kidneys. It is also made in plants, though its function in plants is not clear. Conversion of dopamine to norepinephrine (Figure 6.157) requires vitamin C.

Dopamine is a neurotransmitter, being released by one nerve cell and then traveling across a synapse to signal an adjacent nerve cell. Dopamine plays a major role in the brain's reward-mediated behavior. Rewards, such as food or social interaction, increase dopamine levels in the brain, as do addictive drugs. Other brain dopamine pathways are involved in motor control and in managing the release of various hormones.

Chemical messenger

Outside the nervous system, dopamine is a local chemical messenger. In blood vessels, it inhibits norepinephrine release and causes vasodilation. In the kidneys, it increases sodium excretion and urine output. It reduces gastrointestinal motility and protects intestinal mucosa in the digestive system and in the immune system, it reduces lymphocyte activity. The effect dopamine has on the pancreas is to reduce insulin production. With the exception of the blood vessels, dopamine is synthesized locally and exerts its effects near the cells that release it.

Epinephrine

Epinephrine (also called adrenalin) is a catecholamine chemically related to norepinephrine that is a hormone with medical applications. It is used to treat anaphylaxis, cardiac arrest, croup, and, in some cases, asthma, when other treatments are not working, due to its ability to favor bronchodilation.

Epinephrine is the drug of choice for treating anaphylaxis. The compound may be given through inhalation, by intravenous injection, or subcutaneous injection and exerts effects through the α- and β-adrenergic receptors. In the body, it is produced and released by adrenal glands and some neurons.

Effects

Physiological effects of epinephrine may include rapid heart beat, increased blood pressure, heart output, pupil dilation, blood sugar concentration and increased sweating. Other physical effects may include shakiness, increased anxiety, and an abnormal heart rhythm.

Norepinephrine

Norepinephrine (also called noradrenalin) is a catecholamine molecule that acts as a hormone and neurotransmitter. It is chemically similar to epinephrine, differing only in the absence of a methyl group on its amine. Norepinephrine is made and released by the central nervous system (locus coeruleus of the brain) and the sympathetic nervous system. The compound is released into the blood stream from adrenal glands and affects α- and β-adrenergic receptors.

Norepinephrine is at its lowest levels during sleep and at its highest levels during stress (fight or flight response). The primary function of norepinephrine is to prepare the body for action. It increases alertness, enhances memory functions, and helps to focus attention. Norepinephrine increases heart rate and blood pressure, increases blood glucose and blood flow to skeletal muscle and decreases flow of blood to the gastrointestinal system.

Medical considerations

Norepinephrine may be injected to overcome critically low blood pressure and drugs countering its effects are used to treat heart conditions. α-blockers, for example, are used to battle cardiovascular and psychiatric disorders. β-blockers counter a different set of norepinephrine's effects than α-blockers and are used to treat glaucoma, migraine headaches and other cardiovascular problems.

Pyruvate family

The family of amino acids derived from pyruvate has four members, each with a simple aliphatic side chain no longer than four carbons. The simplest of these is alanine.

Alanine

Alanine is the amino acid that is most easily produced from pyruvate. The simple transamination catalyzed by alanine transaminase produces alanine from pyruvate.

Alternative pathways for synthesis of alanine include catabolism of valine, leucine, and isoleucine.

Glucose-alanine cycle

The glucose-alanine cycle is an important nitrogen cycle related to the Cori cycle that occurs between muscle and liver cells in the body (see HERE). In it, breakdown of glucose in muscles leads to pyruvate. When nitrogen levels are high, pyruvate is transaminated to alanine, which is exported to hepatocytes.

In the liver cells, the last transamination of the glucose-alanine cycle occurs. The amine group of alanine is transferred to α-ketoglutarate to produce pyruvate and glutamate. Glucose can then be made by gluconeogenesis from pyruvate. Importantly, breakdown of glutamate yields ammonium ion, which can be made into urea for excretion, thus reducing the body's load of potentially toxic amines. This pathway may be particularly important in the brain.

Another way of removing excess ammonium from a tissue is by attaching it to glutamate to make glutamine. Glutamate is a neurotransmitter, so having an alternative way of removing amines (glucose-alanine cycle) is important, especially in the brain.

Leucine

Like valine and isoleucine, leucine is an essential amino acid in humans. In adipose tissue and muscle, leucine is used in sterol synthesis. It is the only amino acid to stimulate muscle protein synthesis, and as a dietary supplement in aged rats, it slows muscle degradation. Leucine is an activator of mTOR, a protein which, when inhibited, has been shown to increase life span in Saccharomyces cerevisiae, C. elegans, and Drosophila melanogaster.

Metabolism of leucine, valine, and isoleucine (also called Branched Chain Amino Acids - BCAAs) starts with decarboxylation of pyruvate and attachment of the two-carbon hydroxyethyl fragment to thiamine pyrophosphate (Figure 6.161). Metabolism of isoleucine proceeds with attachment of the hydroxylated two carbon piece (hydroxyethyl-TPP) to α-ketobutyrate and is covered in the section describing that amino acid (see HERE).

Metabolism of valine and leucine proceeds with attachment of the hydroxyethyl piece from TPP to another pyruvate to create α-acetolactate. Rearrangement of α-acetolactate by acetolactate mutase makes 3-hydroxy-3-methyl-2-oxobutanoate.

Reduction with NAD(P)H by acetohydroxy acid isomeroreductase yields α,β-dihydroxyisovalerate.

Loss of water, catalyzed by dihydroxyacid dehydratase produces α-ketoisovalerate.

This molecule is a branch point for synthesis of leucine and valine. Addition of an acetyl group from acetyl-CoA yields α-isopropylmalate (catalyzed by α-isopropylmalate synthase).

Rearrangement, catalyzed by isopropylmalate dehydratase, gives rise to β-isopropylmalate.

Oxidation by isopropylmalate dehydrogenase and NAD+, gives α-ketoisocaproate.

Transamination of it (catalyzed by leucine aminotransferase and using glutamate) gives the final product of leucine (top of next column).

Valine

An essential amino acid in humans, valine is derived in plants from pyruvate and shares part of its metabolic synthesis pathway with leucine and a small slice of it with isoleucine. Metabolism of all three amino acids starts with decarboxylation of pyruvate and attachment of the two-carbon hydroxyethyl fragment to thiamine pyrophosphate (Figure 6.161), as noted above.

As seen earlier, α-ketoisovalerate is the molecule at the point in the metabolic pathway where synthesis of valine branches from that of leucine. In fact, α-ketoisovalerate is only one step away from valine. Transamination of α-ketoisovalerate catalyzed by valine isoleucine aminotransferase gives valine.

Isoleucine

Synthesis of isoeleucine (an essential amino acid in humans) begins in plants and microorganisms with pyruvate and α-ketobutyrate (a byproduct of threonine metabolism - threonine deaminase - Figure 6.162).

Metabolism of isoleucine proceeds with attachment to α-ketobutyrate of the hydroxyethyl-TPP product of pyruvate decarboxylation to form α-aceto-α-hydroxybutyrate. The reaction is catalyzed by acetolactate synthase. Rearrangement and reduction by acetohydroxy acid isomeroreductase and NAD(P)H yields α,β-dihydroxy-β-methylvalerate. Shown on next page.

Loss of water (catalyzed by dihydroxy acid dehydratase) gives α-keto-β-methylvalerate.

Transamination (using glutamate and valine isoleucine transaminase) yields isoleucine.

Interestingly, several of the enzymes of valine metabolism catalyze reactions in the isoleucine pathway. Though the substrates are slightly different, they are enough like the valine intermediates that they are recognized as substrates.

Isoleucine has a second asymmetric center within it, but only one isomeric form of the four possible ones from the two centers is found biologically.

Regulation of synthesis

Regulation of synthesis of the branched chain amino acids (BCAAs - valine, leucine, and isoleucine) is complex. The key molecule in the regulation is α-ketobutyrate, which is synthesized in cells as a breakdown product of threonine. The enzyme catalyzing its synthesis is threonine deaminase (Figure 6.162), which is allosterically regulated. The enzyme is inhibited by its own product (isoleucine) and activated by valine, a product of a parallel pathway.

Thus, when valine concentration is high, the balances shifts in favor of production of isoleucine and since isoleucine competes with valine and leucine for hydroxyethyl-TPP, synthesis of these two amino acids goes down. When isoleucine concentration increases, threonine deaminase is inhibited, shifting the balance back to production of valine and leucine.

Attenuation

Another control mechanism for regulation of leucine synthesis occurs in bacteria and is known as attenuation. In this method, accumulation of leucine speeds the process of translation of a portion of the mRNA copy of the leucine operon (coding sequences for enzymes necessary to make leucine). This, in turn, causes transcription of the genes of the leucine operon to terminate prematurely, thus stopping production of the enzymes necessary to make leucine.

When leucine levels fall, translation slows, preventing transcription from terminating prematurely and allowing leucine metabolic enzymes to be made. Thus, leucine levels in the cell control the synthesis of enzymes necessary to make it.

Histidine family

Synthesis of histidine literally occurs in a class by itself - there are no other amino acids in its synthesis family. The amino acid is made in plants (Arabidopsis, in this case) by a pathway that begins with ribose-5-phosphate. The overall pathway is show in the green text boxes on the next two pages. Abbreviations used in the boxes are shown below.

Enzyme names

1 = Ribose-phosphate diphosphokinase
2= ATP-phosphoribosyltransferase
3 = Phosphoribosyl-ATP pyrophospohydrolase
4 = Phosphoribosyl-AMP cyclohydrolase
5 = ProFAR-I (N'-[(5'phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide isomerase)
6 = Imidazole glycerol-phosphate synthase (IGPS)
7 = Imidazole glycerol-phosphate dehydratase
8 = Histidinol-phosphate aminotransferase
9 = Histidinol-phosphate phosphatase
10 = Histidinol dehydrogenase

Abbreviations used

1 - PRPP = Phosphoribosyl Pyrophosphate
2. PRATP = Phosphoribosyl ATP
3. PRAMP = Phosphoribosyl AMP
4. ProFAR = (N′-[(5′-phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide) ribonucleotide
5. PRFAR = (N′-[(5-phosphoribulosyl)formimino]-5-aminoimidazole-4-carboxamide) ribonucleotide
6. IGP = Imidazole glycerol-phosphate
7. AICAR = 5′-phosphoribosyl-4-carboximide-5-aminoimidazole
8. IAP = Imidazole acetol-phosphate
9. α-KG = α-ketoglutarate

Histidine is a feedback inhibitor of ATP-phosphoribosyltransferase and thus helps to regulate its own synthesis. Histidine is the only amino acid to contain an imidazole ring. It is ionizable and has a pKa of about 6. As a result, histidine's R-group can gain/lose a proton at pH values close to cellular conditions.

Selenocysteine

A cysteine analog commonly referred to as the 21st amino acid, selenocysteine (Figure 6.163) is an unusual amino acid occasionally found in proteins. Although it is rare, selenocysteine has been found in proteins in bacteria, archaea and eukaryotes.

In contrast to amino acids such as phosphoserine, hydroxyproline, or acetyl-lysine, which arise as a result of post-translational modifications, selenocysteine is actually built into growing peptide chains in ribosomes during the process of translation.

No codon specifies selenocysteine, so to incorporate it into a protein, a tRNA carrying it must bind to a codon that normally specifies STOP (UGA). This alternative reading of the UGA is dependent on formation of a special hairpin loop structure in the mRNA encoding selenoproteins.

Selenium is rather toxic, so cellular and dietary concentrations are typically exceedingly low. About 25 human proteins are known to contain the amino acid. These include five glutathione peroxidases, and three thioredoxin reductases. Iodothyronine deiodinase, a key enzyme that converts thyroxine to the active T3 form, also contains selenocysteine in its active site. All of these proteins contain a single selenocysteine.

A eukaryotic protein known as selenoprotein P, found in the blood plasma of animals, contains ten selenocysteine residues and is thought to function as an antioxidant and/or in heavy metal detoxification. Besides selenocysteine, at least two other biological forms of a seleno-amino acid are known. These include 1) selenomethionine (Figure 6.164), a naturally occurring amino acid in Brazil nuts, cereal grains, soybeans, and grassland legumes and 2) methylated forms of selenocysteine, such as Se-methylselenocysteine, are found in Astragalus, Allium, and Brassica species.

Stop codon

The specifics of the process of translation will be described elsewhere in the book, but to get selenocysteine into a protein, the tRNA carrying selenocysteine pairs with a stop codon (UGA) in the mRNA in the ribosome. Thus, instead of stopping translation, selenocysteine can incorporated into a growing protein and translation continues instead of stopping.

Four genes are involved in preparation of selenocysteine for incorporation into proteins. They are known as sel A, sel B, sel C, and sel D. Sel C codes for the special tRNA that carries selenocysteine. The amino acid initially put onto the selenocysteine tRNA is not selenocysteine, but rather serine. Action of sel A and sel D are necessary to convert the serine to a selenocysteine.

An intermediate in the process is selenophosphate, which is the selenium donor. It is derived from H2Se, the form in which selenium is found in the cell. The tRNA carrying selenocysteine has a slightly different structure than other tRNAs, so it requires assistance in translation. The sel B gene encodes for an EF-Tu-like protein that helps incorporate the selenocysteine into the protein during translation.

Recoding the UGA

Using UGA codons to incorporate selenocysteine into proteins could wreak havoc if done routinely, as UGA, in fact, almost always functions as a stop codon and is only rarely used to code for selenocysteine. Fortunately, there is a mechanism to ensure that the reading of a UGA codon as selenocysteine occurs only when the mRNA encodes a selenoprotein.

Unusual structures in mRNAs

The mRNAs for selenocysteine-containing proteins form unusual mRNA structures around the UGA codon that make the ribosome “miss” it as a stop codon and permit the tRNA with selenocysteine to be incorporated instead.

Pyrrolysine

Like selenocysteine, pyrrolysine is a rare, unusual, genetically encoded amino acid found in some cells. Proteins containing it are enzymes involved in methane metabolism and so far have been found only methanogenic archaeans and one species of bacterium. The amino acid is found in the active site of the enzymes containing it. It is sometimes referred to as the 22nd amino acid.

Synthesis of the amino acid biologically begins with two lysines. One is converted to (3R)-3-Methyl-D-ornithine, which is attached to the second lysine. After elimination of an amine group, cyclization, and dehydration, L-pyrrolysine is produced. Pyrrolysine is attached to an unusual tRNA (pylT gene product) by action of the aminoacyl tRNA synthetase encoded by the pylS gene. This unusual tRNA can pair with the UAG stop codon during translation and allow for incorporation of pyrrolysine into the growing polypeptide chain during translation in a manner similar to incorporation of selenocysteine.

Urea cycle

The urea cycle holds the distinction of being the first metabolic cycle discovered - in 1932, five years before the citric acid cycle. It is an important metabolic pathway for balancing nitrogen in the bodies of animals and it takes place primarily in the liver and kidney.

Organisms, like humans, that excrete urea are called ureotelic. Those that excrete uric acid (birds, for example) are called uricotelic and those that excrete ammonia (fish) are ammonotelic. Ammonia, of course, is generated by metabolism of amines and is toxic, so managing levels of it is critical for any organism. Excretion of ammonia by fish is one reason that an aquarium periodically requires cleaning and replacement of water.

Liver failure can lead to accumulation of nitrogenous waste and exacerbates the problem. As shown in Figure 1.166, the cycle contains five reactions, with each turn of the cycle producing a molecule of urea. Of the five reactions, three occur in the cytoplasm and two take place in the mitochondrion. (The reaction making carbamoyl phosphate, catalyzed by carbamoyl phosphate synthetase is not shown in the figure.)

Ornithine synthesis

Though the cycle doesn’t really have a starting point, a common place to begin discussion is with the molecule of ornithine. As discussed elsewhere in this book, ornithine intersects the metabolic pathways of arginine and proline.

Ornithine is found in the cytoplasm and is transported into the mitochondrion by the ornithine-citrulline antiport of the inner mitochonrial membrane. In the matrix of the mitochondrion, two reactions occur relevant to the cycle. The first is formation of carbamoyl phosphate from bicarbonate, ammonia, and ATP catalyzed by carbamoyl phosphate synthetase I.

Carbamoyl phosphate then combines with ornithine in a reaction catalyzed by ornithine transcarbamoylase to make citrulline.

The citrulline is transported out to the cytoplasm by the ornithine-citrulline antiport mentioned above. In the cytoplasm, citrulline combines with L-aspartate using energy of ATP to make citrullyl-AMP (an intermediate) followed by argininosuccinate. The

reaction is catalyzed by argininosuccinate synthase.

Next, fumarate is split from argininosuccinate by argininosuccinate lyase to form arginine.

Water is used by arginase to cleave arginine into urea and ornithine, completing the cycle.

Urea is less toxic than ammonia and is released in the urine. Some organisms make uric acid for the same reason.

It is worth noting that aspartic acid, ammonia, and bicarbonate enter the cycle and fumarate and urea are produced by it. Points to take away include 1) ammonia is converted to urea using bicarbonate and the amine from aspartate; 2) aspartate is converted to fumarate which releases more energy than if aspartate were converted to oxaloacetate, since conversion of fumarate to malate to oxaloacetate in the citric acid cycle generates an NADH, but direct conversion of aspartate to oxaloacetate does not; and 3) glutamate and aspartate are acting as shuttles to funnel ammonia into the cycle. Glutamate, as will be seen below, is a scavenger of ammonia.

Urea cycle regulation

The urea cycle is controlled both allosterically and by substrate concentration. The cycle requires N-acetylglutamate (NAG) for allosteric activation of carbamoyl phosphate synthetase I. The enzyme that catalyzes synthesis of NAG, NAG synthetase, is activated by arginine and glutamate. Thus, an indicator of high amine levels, arginine, and an important shuttler of amine groups, glutamate, stimulates the enzyme that activates the cycle.

The reaction catalyzed by NAG synthetase is

At the substrate level, all of the other enzymes of the urea cycle are controlled by the concentrations of substrates they act upon. Only at high concentrations are the enzymes fully utilized.

Complete deficiency of any urea cycle enzyme is fatal at birth, but mutations resulting in reduced expression of enzymes can have mixed effects. Since the enzymes are usually not limiting for these reactions, increasing substrate can often overcome reduced enzyme amounts to a point by simply fully activating enzymes present in reduced quantities.

Ammonia accumulation

However, if the deficiencies are sufficient, ammonium can accumulate and this can be quite problematic, especially in the brain, where mental deficiencies or lethargy can result. Reduction of ammonium concentration relies on the glutamate dehydrogenase reaction (named for the reverse reaction).

Additional ammonia can be taken up by glutamate in the glutamine synthetase reaction.

The result of these reactions is that α-ketoglutarate and glutamate concentrations will be reduced and the concentration of glutamine will increase. For the brain, this is a yin/yang situation. Removal of ammonia is good, but reduction of α-ketoglutarate concentration means less energy can be generated by the citric acid cycle. Further, glutamate is, itself, an important neurotransmitter and a precursor of another neurotransmitter - γ-aminobutyric acid (GABA).

Energy generation

From an energy perspective, the urea cycle can be said to break even or generate a small amount of energy, if one includes the energy produced in releasing ammonia from glutamate (one NADH). There are two NADHs produced (including the one for converting fumarate to oxaloacetate), which give 4-6 ATPs, depending on how efficiently the cell performs electron transport and oxidative phosphorylation.

The cycle takes in 3 ATPs and produces 2 ADPs and one AMP. Since AMP is equivalent to 2 ATP, the cycle uses 4 ATP. Thus, the cycle either breaks even in the worst case or generates 2 ATPs in the best case.

Amino acid catabolism

Amino acids are divided according to the pathways involved in their degradation. There are three general categories. Ones that yield intermediates in the glycolysis pathway are called glucogenic and those that yield intermediates of acetyl-CoA or acetoacetate are called ketogenic. Those that involve both are called glucogenic and ketogenic. These are shown in Figures 6.167 and 6.168.

As seen in the two figures, amino acids largely produce breakdown products related to intermediates of the citric acid cycle or glycolysis, but this isn't the complete picture. Some amino acids, like tryptophan, phenylalanine, and tyrosine yield hormones or

neurotransmitters on further metabolism (as noted earlier). Others like cysteine and methionine must dispose of their sulfur and all of the amino acids must rid themselves of nitrogen, which can happen via the urea cycle, transamination, or both.

Tyrosine catabolism

Breakdown of tyrosine (Figure 6.169) is a five step process that yields acetoacetate and fumarate. Enzymes involved include 1) tyrosine transaminase; 2) p-hydroxylphenylpyruvate dioxygenase; 3) homogentisate dioxygenase; 4) maleylacetoacetate cis-trans-isomerase; and 5) 4-fumaryl acetoacetate hydrolase.

Breakdown of leucine is a multi-step process ultimately yielding the ketone body acetoacetate and acetyl-CoA. Branched chain amino acids (BCAAs - valine, leucine, and isoleucine) rely on Branched Chain AminoTransferase (BCAT) followed by Branched Chain α-ketoacid dehydrogenase (BCKD) for catabolism.

Breakdown of isoleucine yields intermediates that are both ketogenic and glucogenic. These include acetyl-CoA and propionyl-CoA.

Breakdown of valine is a multi-step process ultimately yielding propionyl-CoA.

This page titled 6.6: Amino Acids and the Urea Cycle is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

6.7: Other Lipids

Source: BiochemFFA_6_4.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

Sugars are the building blocks of carbohydrates, amino acids are the building blocks of proteins and nucleotides are the building blocks of the nucleic acids - DNA and RNA. Another crucial building block is **acetyl-CoA**, which is used to build many lipid substances, including fatty acids, cholesterol, fat soluble vitamins, steroid hormones, prostaglandins, endocannabinoids, and the bile acids. Indeed, acetyl-CoA goes into more different classes of molecule than any other building block.

Isoprenoids

We focus our attention here on a group of molecules made from acetyl-CoA that are known as as the isoprenoids. Isoprenoids are a large, diverse and ancient group of molecules that are found in all three domains of life. As noted earlier, they are components of membrane lipids in the cell membranes of archaebacteria, but beyond this, they serve an astonishing variety of functions. From photosynthetic pigments to plant defense compounds, from flavor compounds in cinnamon, mint, ginger and cloves to plant and animal hormones, from the cannabinoids in marijuana to the lycopene that gives tomatoes their color, and from heme to the quinones in the electron transport chain, isoprenoids are ubiquitous in cells. Isoprenoids derive their name from the fact that they are, in fact, made from five carbon building blocks called isoprenes that are derived from acetyl-CoA. The synthesis of the two isoprene units - isopentenyl pyrophosphate and dimethylallyl pyrophosphate is shown in Figure 6.111 and Figure 6.112.

The pathway leading up to isoprene synthesis overlaps with that of ketone body synthesis, for the two reactions (Figure 6.112), as has been discussed earlier in this book (see HERE). Thiolase catalyzes the initial reaction, joining together two acetyl-CoA molecules to make acetoacetyl-CoA. In the second reaction catalyzed by HMG-CoA synthase, a third acetyl-CoA is joined to form the six carbon compound known as hydroxymethyl glutaryl-CoA (HMG-CoA). Reaction three is an important one biologically and medically because of the enzyme catalyzing it - HMG-CoA reductase.

Statins

Medically, HMG-CoA reductase is the target of a class of drugs known as statins (Figure 6.113 & Movie 6.1), which are used to reduce cholesterol levels in people. These competitive inhibitors, which compete with HMG-CoA for binding have two effects. First, they reduce the production of mevalonate, which restricts the amount of substrate available to make cholesterol. Second, and perhaps more importantly, they increase production of LDL receptors in the liver, which favors uptake and destruction of LDLs, thus lowering serum cholesterol levels.

Regulation

Biologically, the HMG-CoA reductase enzyme is also of importance because it is the primary regulatory point in cholesterol synthesis. Control of it is complex. First, it is feedback inhibited by cholesterol itself. High levels of glucose in the blood activate the enzyme. Phosphorylation by AMP-activated protein kinase inhibits its activity. Interestingly, the same enzyme phosphorylates and inactivates acetyl-CoA carboxylase - the only regulatory enzyme controlling fatty acid synthesis. Transcription of the gene encoding HMG-CoA reductase

is enhanced by binding of the sterol regulatory element binding protein (SREBP) to the sterol recognition element (SRE) located near the gene coding sequence. As cholesterol levels rise, SREBP is proteolytically cleaved and transcription stops.

From HMG-CoA, the enzyme HMG-CoA reductase catalyzes the formation of mevalonate. This reaction requires NADPH and results in release of coenzyme A. Mevalonate gets phosphorylated twice and then decarboxylated to yield the five carbon intermediate known as isopentenyl-pyrophosphate (IPP). IPP is readily converted to the other important isoprenoid unit, dimethylallylpyrophosphate (DMAPP).

Isoprenes

These two five carbon compounds, IPP and DMAPP, are also called isoprenes (Figure 6.115) and are the building blocks for the synthesis of cholesterol and related compounds. This pathway proceeds in the direction of cholesterol starting with the joining of

IPP and DMAPP to form geranyl-pyrophosphate. Geranyl-pyrophosphate combines with another IPP to make farnesyl-pyrophosphate, a 15-carbon compound.

Squalene

Two farnesyl-pyrophosphates join to create the 30-carbon compound known as squalene. Squalene, in a complicated rearrangement involving reduction and molecular oxygen forms a cyclic intermediate known as lanosterol (Figure 6.116) that resembles cholesterol. Conversion of lanosterol to cholesterol is a lengthy process involving 19 steps that occur in the endoplasmic reticulum.

The cholesterol biosynthesis pathway from lanosterol is a long one and requires significant amounts of reductive and ATP energy. As noted earlier (see HERE), cholesterol has an important role in membranes. It is also a precursor of steroid hormones and bile acids and its immediate metabolic precursor, 7-dehydrocholesterol (Figure 6.117), branches to form vitamin D (Figure 6.118).

All steroid hormones in animals are made from cholesterol and include the progestagens, androgens, estrogens, mineralocorticoids, and the glucocorticoids. The branch molecule for all of the steroid hormones is the cholesterol metabolite (and progestagen) known as pregnenalone (Figure 6.119). The progestagens are thus precursors of all of the other classes of steroid hormones.

The estrogens are derived from the androgens in an interesting reaction that required formation of an aromatic ring (Figure 6.120). The enzyme catalyzing this reaction is known as an aromatase and it is of medical significance. The growth of some tumors is stimulated by estrogens, so aromatase inhibitors are prescribed to prevent the formation of estrogens and slow tumor growth. Two commonly used inhibitors include exemestane (a suicide inhibitor - Figure 6.121) and anastrozole (a competitive inhibitor).

Other fat-soluble vitamins

Synthesis of other fat soluble vitamins and chlorophyll also branches from the isoprenoid synthesis pathway at geranyl pyrophosphate. Joining of two geranylgeranyl pyrophosphates occurs in plants and bacteria and leads to synthesis of lycopene, which, in turn is a precursor of β-carotene, the final precursor of Vitamin A (see below also). Vitamins E and K, as well as chlorophyll are all also synthesized from geranylgeranyl pyrophosphate.

Bile acid metabolism

Another metabolic pathway from cholesterol leads to the polar bile acids, which are important for the solubilization of dietary fat during digestion. Converting the very non-polar cholesterol to a bile acid involves oxidation of the terminal carbon on the side chain off the rings. Other alterations to increase the polarity of these compounds include hydroxylation of the rings and linkage to other polar compounds.

Common bile acids include cholic acid, chenodeoxycholic acid, glycocholic acid, taurocholic acid, and deoxycholic acid (Figure 6.123). Another important consideration about bile acids is that their synthesis reduces the amount of cholesterol available and promotes uptake of LDLs by the liver. Normally bile acids are recycled efficiently resulting in limited reduction of cholesterol levels. However, inhibitors of the recycling promote reduction of cholesterol levels.

Vitamin A Synthesis

Vitamin A is important for many cellular functions related to growth, differentiation and organogenesis during embryonic development, tissue maintenance, and vision, to name a few.

There are three main active forms of the vitamin, retinal, retinol and retinoic acid, each with its own set of functions. Retinal, complexed with the protein, opsin, is found in the rod cells of the retina and is necessary for vision. Retinol and retinoic acid both function as signaling molecules that can modulate gene expression during development.

Synthesis of vitamin A occurs as a branch in synthesis of isoprenoids. Addition of isopentenyl pyrophosphate to farnesyl pyrophosphate creates a 20-carbon intermediate, geranylgeranyl pyrophosphate (GGPP - Figure 6.124).

Joining of two GGPPs creates a 40 carbon intermediate that is unstable and decomposes to phytoene. Desaturases oxidize two single bonds in phytoene, creating lycopene.

Lycopene is a linear 40 carbon unsaturated molecule found in tomatoes and other red vegetables and it gives them their color. Cyclization of end portions of lycopene give rise to β-carotene, the precursor of vitamin A (retinal/retinol - Figure 6.124).

β-carotene is found in carrots and other orange vegetables, and is converted in the body to vitamin A. Catalytic action by β-Carotene 15,15' monooxygenase cleaves β-carotene to form retinal (the aldehyde form used in vision).

The enzyme retinol dehydrogenase catalyzes reduction of retinal to retinol (storage form). Oxidation of retinal creates another important retinoid known as retinoic acid. This form of vitamin A cannot be reduced back to retinal and thus cannot be used for vision or storage.

Instead, retinoic acid has roles in embryonic development. Retinoic acid acts through binding to the Retinoic Acid Receptor (RAR). RAR binds to DNA and affects transcription of several important sets of genes important for differentiation. These include the Hox genes, which control anterior/posterior patterning in early embryonic development.

Sphingolipid synthesis

Synthesis of sphingolipids, which are found primarily in brain and nerve tissue, begins with palmitoyl-CoA and serine that combine to make an 18-carbon amine called 3-keto-sphinganine (Figure 6.125). Reduction of that by NADPH yields dihydrosphingosine and addition of a fatty acid from an acyl-CoA yields N-acylsphinganine, which is a ceramide (Figure 6.126). A ceramide can be converted into a cerebroside by addition of a glucose from UDP-glucose (Figure 6.127).

If a few other simple sugars are added to the cerebroside, a globoside is created. If, instead of adding sugar, a phosphocholine is added from phosphatidylcholine, then sphingomyelin is created (Figure 6.127). If a complex set of sugars are added to to a cerebroside, then a ganglioside results (Figure 6.127).

Sphingolipid breakdown

In the overall metabolism of sphingolipids, the greatest problems arise with their catabolism. Figure 6.128 illustrates the numerous genetic diseases arising from mutations in DNA coding for some of these enzymes. All are lysosomal storage diseases and many of these are quite severe. GM1 glandiosidoses (arising from inability to breakdown GM1 gangliosides) cause severe neurodegeneration and seizures. Individuals suffering from them typically die by age 3. Tay-Sachs disease usually causes death by age 4, though late-onset forms of the disease in adults are known.

With Gaucher's disease, three different types have been described with widely varying effects. In some, the disease is fatal by age four and in others, it does not manifest until teens or even adulthood. Fabry's disease patients can live into their 50s, on average.

Glycerophospholipid metabolism

Glycerophospholipids are the major components of membranes. Synthesis of glycerophospholipids begins with glycerol-3-phosphate. In the first reaction, glycerol-3-phosphate gains a fatty acid at position one from an acyl-CoA, followed by a duplicate reaction at position two to make phosphatidic acid (Figure 6.129). This molecule, which can branch to other reactions to form fats, is an important intermediate in the synthesis of many glycerophospholipids. Glycerophospholipid compounds can often be made by more than one pathway. The nucleotide CDP plays an important role in glycerophospholipid synthesis, serving as part of an activated intermediate for synthesis of phosphatidyl compounds. This is necessary, because formation of the phosphodiester bonds of these compounds requires higher energy input.

Cells use two strategies to accomplish this. Both involve CDP. In the first, CTP combines with phosphatidic acid to make CDP-diacylglcyerol with release of a pyrophosphate. The reaction is catalyzed by phosphatidate cytidylyltransferase.

CDP-diacylglycerol then serves as an activated intermediate to donate the phosphotidate part of itself to another molecule. The reaction below illustrates one example

The second strategy is to make a CDP derivative of the group being added to phosphatidic acid. An example is shown next

Then the CDP donates the phosphocholine to a diacylglycerol to made phosphatidylcholine and CMP

Synthesis of other important glycerophospholipids follows from these basic strategies. Phosphatidylethanolamine can be easily made from phosphatidylserine by decarboxylation.

Phosphatidylethanolamine can serve as a precursor in an alternative pathway for making phosphatidylcholine (SAM = S-Adenosyl Methioinine / SAH = S-Adenosyl Homocysteine)

Phosphatidylserine and phosphatidylethanolamine can swap groups reversibly in the reaction below

Similarly, phosphatidylserine and phosphatidylcholine can be interchanged as follows:

Phosphatidylglycerol can be made from glycerol-3-phosphate and CDP-diacylglycerol

Cardiolipin, which is essentially a diphosphatidyl compound can be made by joining CDP-diacylglyerol with phosphatidylglycerol

Phosphtidylinositol can be made from CDP-diacylglycerol and inositol .

Heme synthesis

The porphyrin ring found in the hemes of animals, fungi, and protozoa (Figure 6.130) is synthesized starting from very simple compounds (Figure 6.131). The process is a bit complicated, occurring between the cytoplasm and the mitochondrion. The first step is the creation of δ-aminolevulinic acid (also called aminolevulinic acid or dALA) from glycine and Succinyl-CoA.

Joining of two δ-aminolevulinic acid molecules together with splitting out of two molecules of water yields porphobilinogen.

Joining of four molecules of porphobilinogen together yields hydroxylmethylbilane (Figure 6.132).

Next, a series of reactions involving 1) loss of water; 2) loss of four molecules of carbon dioxide; 3) loss of two more carbon dioxides, loss of six protons and electrons and (finally) 4) addition of Fe++ with loss of two protons yields heme. Individual heme molecules may be further processed.

Two enzymes in heme synthesis are sensitive to the presence of lead, and this is one of the primary causes of lead toxicity in humans. Inhibition of the enzymes leads to 1) anemia and 2) accumulation of δ-aminolevulinic acid, which can be harmful to neurons in development, resulting in learning deficiencies in children.

Porphyria

Defects in enzymes of the pathway can also lead to porphyrias, diseases in which one or more of the intermediates in the heme synthesis pathway accumulate due to deficiency of the enzyme necessary to convert the accumulating material into the next molecule in the pathway. The accumulation of purplish intermediates gave the diseases the name porphyria from the Greek word for purple.

Severe porphyrias can lead to brain damage, nerve damage, and mental disturbances. The “madness” of King George III may have been due to a form of porphyria. In other manifestations of the disease, cutaneous porphyrias cause skin problems on exposure to light. This need, for patients with certain forms of porphyria, to avoid light, coupled with the fact that porphyrias can be treated by blood tranfusions, may have led to the legend of vampires.

Breakdown of heme

Catabolism of heme (Figure 6.133) begins in macrophages within the spleen . Targets for degradation are hemes within damaged red blood cells, which get removed from the blood supply due to their appearance. It is because of this system, for example, that sickle cell anemia is classified as an anemia (decrease in red blood cells or hemoglobin in the blood). After cells have sickled, they lose their shape and are more likely to be removed from the blood by this process, leaving the patient weakened from low blood cell counts.

The first biochemical step in catabolism is conversion of heme to biliverdin. This reaction is catalyzed by heme oxygenase and requires electrons from NADPH. In the process, Fe++ is released. Interestingly, carbon monoxide is also produced and it acts as a vasodilator.

Next, biliverdin is converted to bilirubin by biliverdin reductase and is secreted from the liver into bile. Bacteria in the intestine convert bilirubin to urobilinogens, some of which is absorbed intestinal cells and transported into kidneys and excreted. The yellow color of urine arises from the compound known as urobilin, which is an oxidation product of urobilinogen. The remainder of the urobilinogens are converted in the intestinal tract to stercobilinogen whose oxidation product is stercobilin and it gives the color associated with feces.

This page titled 6.7: Other Lipids is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **6.4: Other Lipids** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

6.8: Energy - Photophosphorylation

Source: BiochemFFA_5_3.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy



Photophosphorylation

The third type of phosphorylation to make ATP is found only in cells that carry out photosynthesis. This process is similar to oxidative phosphorylation in several ways. A primary difference is the ultimate source of the energy for ATP synthesis. In oxidative phosphorylation, the energy comes from electrons produced by oxidation of biological molecules. In photosynthesis, the energy comes from the light of the sun. Photons from the sun interact with chlorophyll molecules in reaction centers in the chloroplasts (Figures **6.8.1** and **6.8.2**) of plants or membranes of photosynthetic bacteria.

The similarities of photophosphorylation to oxidative phosphorylation include:

- a membrane associated electron transport chain
- creation of a proton gradient
- harvesting energy of the proton gradient by making ATP with the help of an ATP synthase.

Some of the differences include :

- the source of the electrons – H2O for photosynthesis versus NADH/FADH2 for oxidative phosphorylation
- direction of proton pumping – into the thylakoid space of the chloroplasts versus outside the matrix of the mitochondrion
- movement of protons during ATP synthesis – out of the thylakoid space in photosynthesis versus into the mitochondrial matrix in oxidative phosphorylation
- nature of the terminal electron acceptor – NADP+ in photosynthesis versus O2 in oxidative phosphorylation.

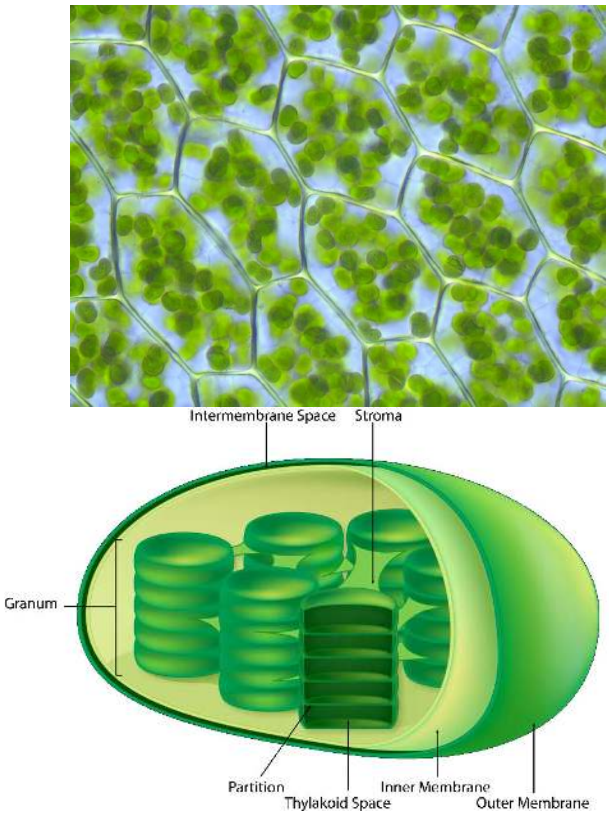


Figure **6.8.2**: *The chloroplast. Image by Aleia Kim*

Electron transport: chloroplasts vs mitochondria

In some ways, the movement of electrons in chloroplasts during photosynthesis is opposite that of electron transport in mitochondria. In photosynthesis, water is the source of electrons and their final destination is NADP+ to make NADPH. In mitochondria, NADH/FADH2 are electron sources and H2O is their final destination. How do biological systems get electrons to go both ways? It would seem to be the equivalent of going to and from a particular place while always going downhill, since electrons will move according to potential.

Solar power

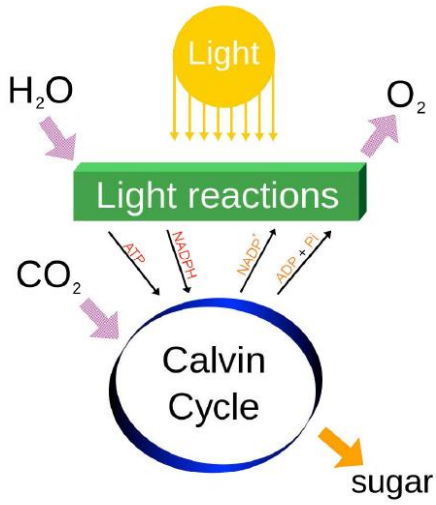


Figure **6.8.3**: *Overview of photosynthesis. Wikipedia*

The answer is the captured energy of the photons from the sun (Figure 5.59), which elevates electrons to an energy where they move “downhill” to their NADPH destination in a Z-shaped scheme. The movement of electrons through this scheme in plants requires energy from photons in two places to “lift” the energy of the electrons sufficiently.

Last, it should be noted that photosynthesis actually has two phases, referred to as the light cycle (described above) and the dark cycle, which is a set of chemical reactions that captures CO2 from the atmosphere and “fixes” it, ultimately into glucose. The dark cycle is also referred to as the Calvin Cycle and is discussed HERE.

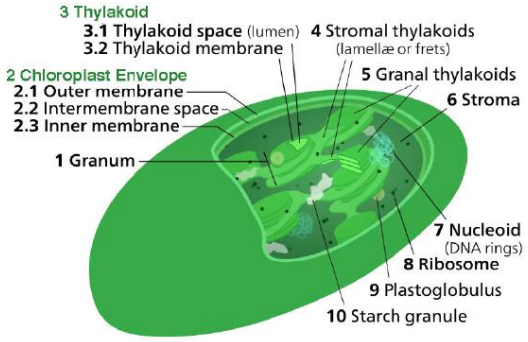


Figure **6.8.4**: *Chloroplast anatomy. Wikipedia*

Photosynthesis

Photosynthesis is an energy capture process found in plants and other organisms to harvest light energy and convert it into chemical energy. This photochemical energy is stored ultimately in carbohydrates which are made using ATP (from the energy harvesting), carbon dioxide and water. In most cases, a byproduct of the process is oxygen, which is released from water in the capture process. Photosynthesis is responsible for most of the oxygen in the atmosphere and it supplies the organic materials and most of the energy used by life on Earth.

Steps

The steps in the photosynthesis process varies slightly between organisms. In a broad overview, it always starts with energy capture from light by protein complexes, containing chlorophyll pigments, called reaction centers. Plants sequester these proteins in chloroplasts, but bacteria, which don’t have organelles, embed them in their plasma membranes.

Energy from the light is used to strip electrons away from electron donors (usually water) and leave a byproduct (oxygen, if water was used). Electrons are donated to a carrier and ultimately are accepted by NADP+, to become NADPH. As electrons travel towards NADP+, they generate a proton gradient across the thylakoid membrane, which is used to drive synthesis of ATP. Thus NADPH, ATP, and oxygen are the products of the first phase of photosynthesis called the light reactions. Energy from ATP and electrons from NADPH are used to reduce CO2 and build sugars, which are the ultimate energy storage directly arising from photosynthesis.

Chloroplasts

Chloroplasts are found in almost all aboveground plant cells, but are primarily concentrated in leaves. The interior of a leaf, below the epidermis is made up of photosynthesis tissue called mesophyll, which can contain up to 800,000 chloroplasts per square millimeter.

The chloroplast’s membrane has a phospholipid inner membrane, a phospholipid outer membrane, and a region between them called the intermembrane space (Figure 5.61). Within the inner chloroplast membrane is the stroma, in which the chloroplast DNA and the enzymes of the Calvin cycle are located. Also within the stroma are stacked, flattened disks known as thylakoids which are defined by their thylakoid membranes. The space within the thylakoid membranes are termed the thylakoid spaces or thylakoid lumen. The protein complexes containing the light-absorbing pigments, known as photosystems, are located on the thylakoid membrane. Besides chlorophylls, carotenes and xanthophylls are also present, allowing for absorption of light energy over a wider range. The same pigments are used by green algae and land plants.

Brown algae and diatoms add fucoxanthin (a xanthophyll) and red algae add phycoerythrin to the mix. In plants and algae, the pigments are held in a very organized fashion complexes called antenna proteins that help funnel energy, through resonance energy transfer, to the reaction center chlorophylls. A system so organized is called a light harvesting complex. The electron transport complexes of photosynthesis are also located on the thylakoid membranes.

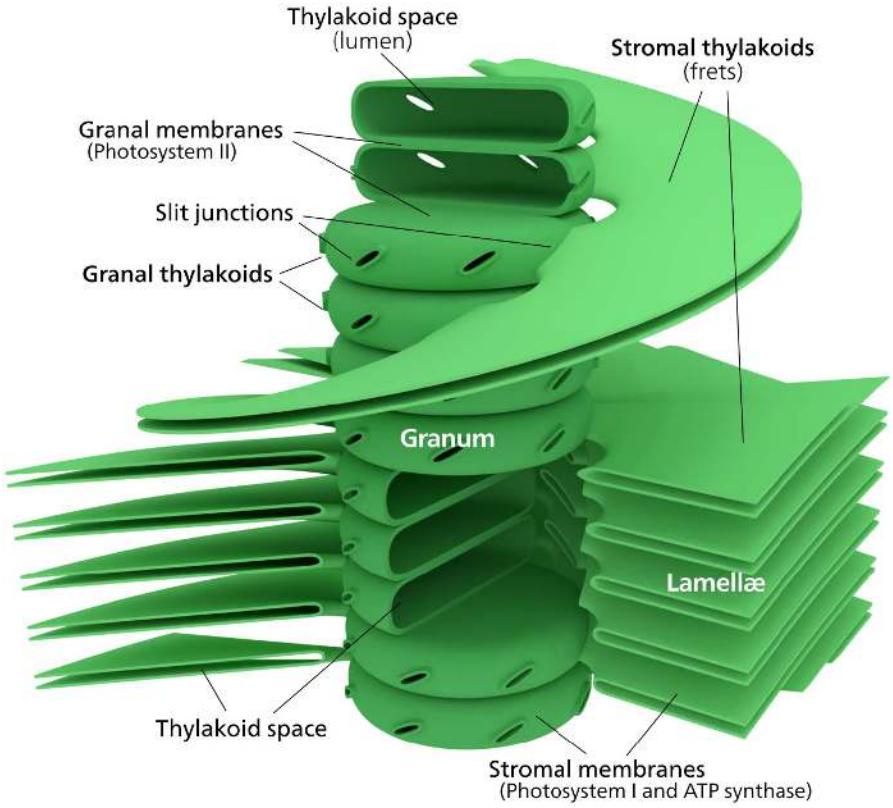


Figure 6.8.5: Side view of thylakoids. Wikipedia

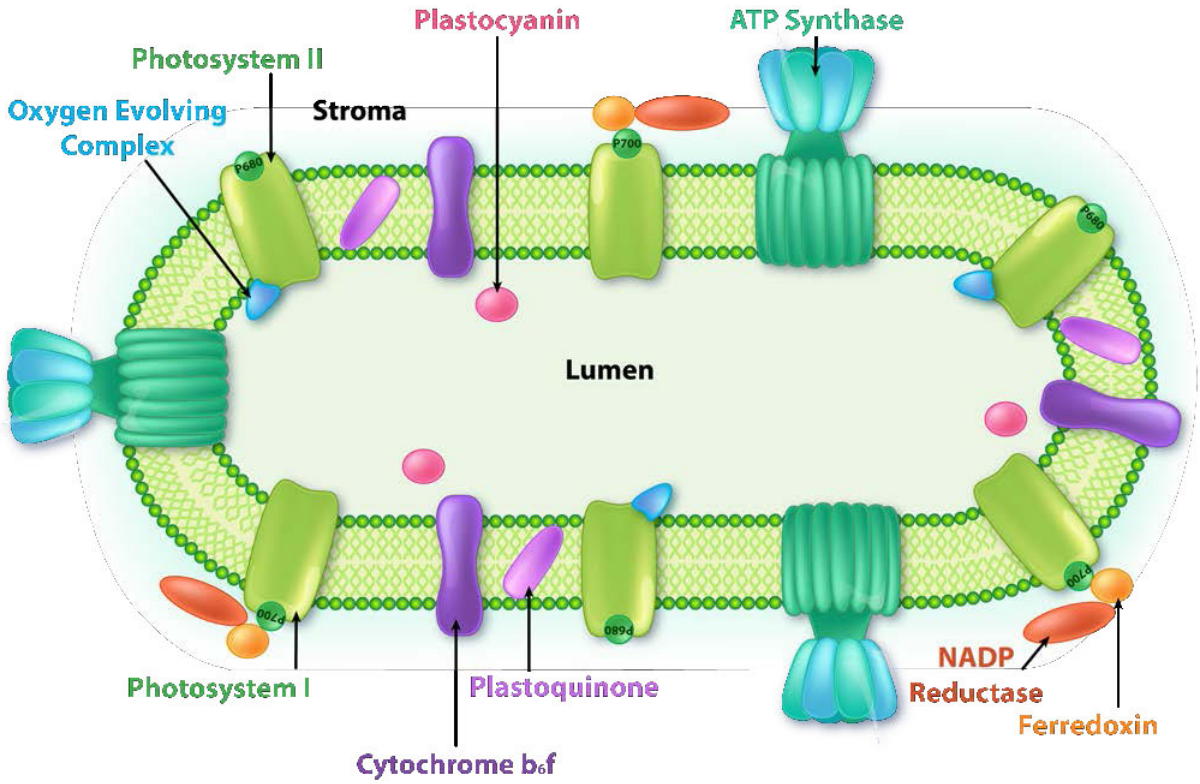


Figure* 6.8.6*: *Complexes in the thylakoid membrane. Image by Aleia Kim*

Light reactions of photosynthesis

In chloroplasts, the light reactions of photosynthesis involving electron transfer occur in the thylakoid membranes (Figure **6.8.6**). Separate biochemical reactions involving the assimilation of carbon dioxide to make glucose are referred to as the Calvin cycle, also sometimes referred to as the “dark reactions”. This will be discussed elsewhere in the section on metabolism (HERE).

The chloroplasts are where the energy of light is captured, electrons are stripped from water, oxygen is liberated, electron transport occurs, NADPH is formed, and ATP is generated. The thylakoid membrane corresponds to the inner membrane of the mitochondrion for transport of electrons and proton pumping (Figure **6.8.4**).

The thylakoid membrane does its magic using four major protein complexes. These include Photosystem II (PS II), Cytochrome b6f complex (Cb6f), Photosystem I (PS I), and ATP synthase. The roles of these complexes, respectively, are to capture light energy, create a proton gradient from electron movement, capture light energy (again), and use proton gradient energy from the overall process to synthesize ATP.

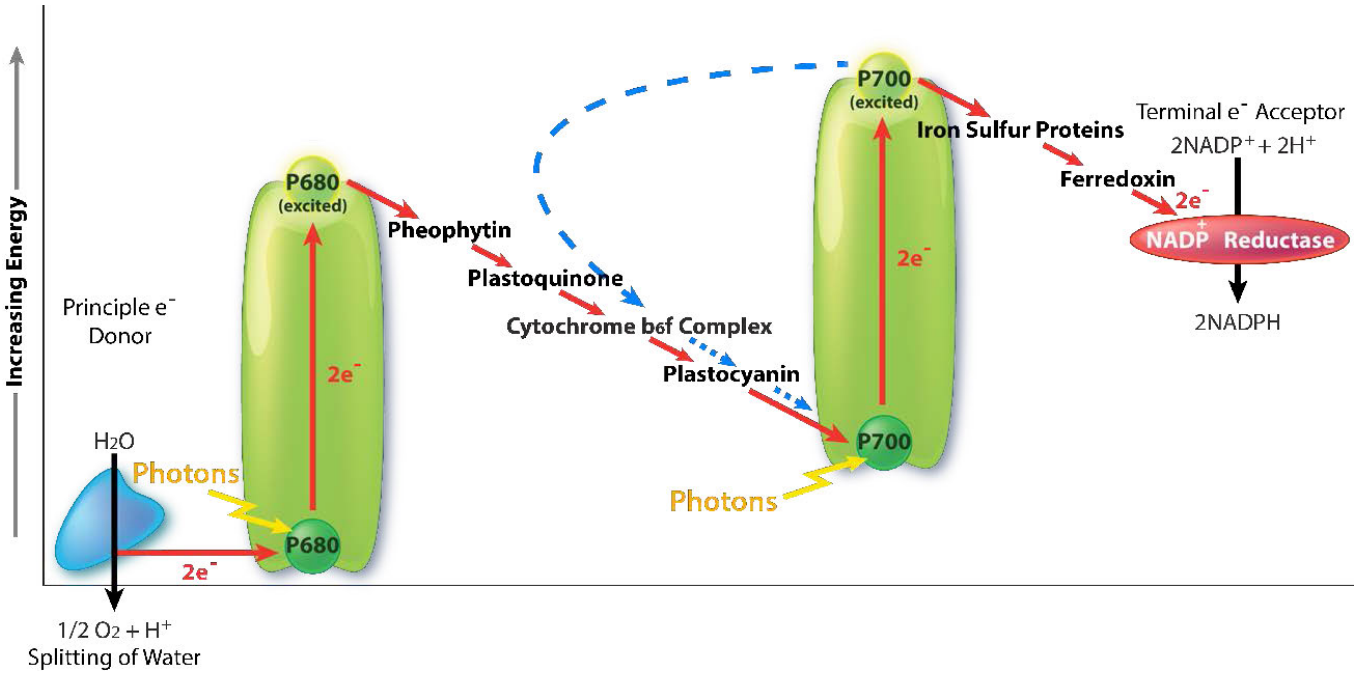


Figure **6.8.7**: Movement of electrons through photosystems. Cyclic photophosphorylation shown by blue dashed line. Image by Aleia Kim and Pehr Jacobson

Light harvesting

Harvesting the energy of light begins in PS II with the absorption of a photon of light at a reaction center. PS II performs this duty best with light at a wavelength of 680 nm and it readily loses an electron to excitation when this occurs, leaving PS II with a positive charge. This electron must be replaced. The ultimate replacement source of electrons is water, but water must lose four electrons and PS II can only accept one at a time.

Manganese centers

An intermediate Oxygen Evolving Complex (OEC) contains four manganese centers that provide the immediate replacement electron that PSII requires. After four electrons have been donated by the OEC to PS II, the OEC extracts four electrons from two water molecules, liberating oxygen and dumping four protons into the thylakoid space, thus contributing to the proton gradient. The excited electron from PS II must be passed to another carrier very quickly, lest it decay back to its original state. It does this, giving its electron within picoseconds to pheophytin (Figure **6.8.8**).

Pheophytin passes the electron on to protein-bound plastoquinones . The first is known as PQA. PQA hands the electron off to a second plastoquinone (PQB), which waits for a second electron and collects two protons to become PQH2, also known as plastoquinol (Figure **6.8.9**). PQH2 passes these to the Cytochrome b6f complex (Cb6f) which uses passage of electrons through it to pump protons into the thylakoid space. ATP synthase makes ATP from the proton gradient created in this way. Cb6f drops the electron off at plastocyanin, which holds it until the next excitation process begins with absorption of another photon of light at 700 nm by PS I.

Absorption of light at PS I

With absorption of a photon of light by PS I, a process begins, that is similar to the process in PS II. PS I gains a positive charge as a result of the loss of an excited electron and pulls the electron in plastocyanin away from it. Meanwhile, the excited electron from PS I passes through an iron-sulfur protein, which gives the electron to ferredoxin (another iron sulfur protein). Ferredoxin then passes the electron off to the last protein in the system known as Ferredoxin:NADP+ oxidoreductase, which gives the electron and a proton to NADP+, creating NADPH.

Note that reduction of NADP+ to NADPH requires two electrons and one proton, so the four electrons and two protons from oxidation of water will result in production of two molecules of NADPH. At this point, the light cycle is complete - water has been

oxidized, ATP has been created, and NADPH has been made. The electrons have made their way from water to NADPH via carriers in the thylakoid membrane and their movement has released sufficient energy to make ATP. Energy for the entire process came from four photons of light.

The two photosystems performing all of this magic are protein complexes that are similar in structure and means of operation. They absorb photons with high efficiency so that whenever a pigment in the photosynthetic reaction center absorbs a photon, an electron from the pigment is excited and transferred to another molecule almost instantaneously. This reaction is called photo-induced charge separation and it is a unique means of transforming light energy into chemical forms.

Cyclic photophosphorylation

Besides the path described above for movement of electrons through PS I, plants have an alternative route that electrons can take. Instead of electrons going through ferredoxin to form NADPH, they instead take a backwards path through the the proton-pumping b6f complex. This system, called cyclic photophosphorylation (Figure **6.8.8**) which generates more ATP and no NADPH, is similar to a system found in green sulfur bacteria. The ability of plants to switch between non-cyclic and cyclic photosystems allows them to make the proper ratio of ATP and NADPH they need for assimilation of carbon in the dark phase of photosynthesis. This ratio turns out to be 3 ATPs to 2 NADPHs.

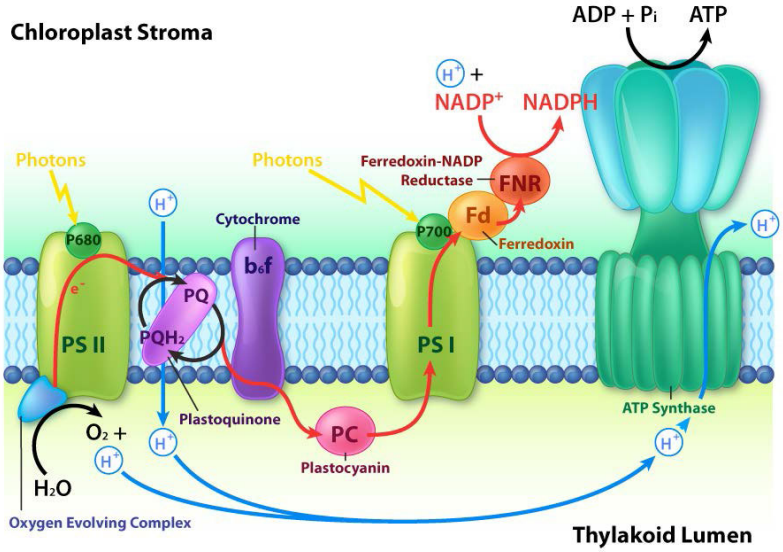


Figure **6.8.8** - Movement of electrons and protons through the thylakoid membrane. Image by Aleia Kim

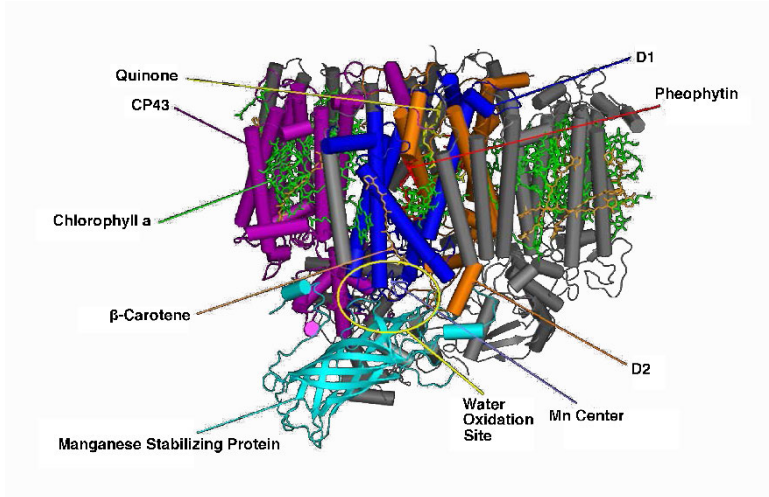


Figure **6.8.9** *- Photosystem II of cyanobacteria. Wikipedia*

Photosynthetic energy

The output of the photophosphorylation part of photosynthesis (O2, NADPH, and ATP), of course, is not the end of the process of photosynthesis. For the growing plant, the NADPH and ATP are used to capture carbon dioxide from the atmosphere and convert it (ultimately) into glucose and other important carbon compounds. This, as noted previously, occurs in the Calvin Cycle (see HERE) in what is called the dark phase of the process. The oxygen liberated in the process is a necessary for respiration of all aerobic life forms on Earth. Indeed, it is believed that essentially all of the oxygen in the atmosphere today is the result the splitting of water in photosynthesis over the many eons that the process has existed.

This page titled 6.8: Energy - Photophosphorylation is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **5.3: Energy - Photophosphorylation** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

7: Regulation of Metabolism and Homeostasis

This page is a placeholder created because the page was deleted, but has sub-pages.

This page titled 7: Regulation of Metabolism and Homeostasis is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

7.1: Signaling

Source: BiochemFFA_7_8.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

Up to this point we have considered how cells carry out biochemical reactions and how they regulate the expression of the genes in response to their internal and external environments. It is intuitively obvious that even unicellular organisms must be able to sense features of their environment, such as the presence of nutrients, if they are to survive. In addition to being able to receive and respond to information from the environment, multicellular organisms must also find ways by which their cells can communicate among themselves.

Coordination

Since different cells take on specialized functions in a multicellular organism, they must be able to coordinate activities. Cells grow, divide, or differentiate in response to specific signals. They may change shape or migrate to another location. At the physiological level, cells in a multicellular organism, must respond to everything from a meal just eaten to injury, threat, or the availability of a mate. They must know when to divide, when to undergo apoptosis (programmed cell death), when to store food, and when to break it down. A variety of mechanisms have arisen to ensure that cell-cell communication is not only possible, but astonishingly swift, accurate and reliable.

How are signals sent between cells? Like pretty much everything that happens in cells, signaling is dependent on molecular recognition. The basic principle of cell-cell signaling is simple. A particular kind of molecule, sent by a signaling cell, is recognized and bound by a receptor protein in (or on the surface of) the target cell. The signal molecules are chemically varied- they may be proteins, short peptides, lipids, nucleotides or catecholamines, to name a few.

Signal properties

The chemical properties of the signal determine whether its receptors are on the cell surface or intracellular. If the signal is small and hydrophobic it can cross the cell membrane and bind a receptor inside the cell. If, on the other hand, the signal is charged, or very large, it would not be able to diffuse through the plasma membrane. Such signals need receptors on the cell surface, typically transmembrane proteins that have an extracellular portion that binds the signal and an intracellular part that passes on the message within the cell (Figure 7.130).

Receptors are specific for each type of signal, so each cell has many different kinds of receptors that can recognize and bind the many signals it receives. Because different cells have different sets of receptors, they respond to different signals or combinations of signals. The binding of a signal molecule to a receptor sets off a chain of events in the target cell. These events could cause change in various ways, including, but not limited to, alterations in metabolic pathways or gene expression in the target cell.

How the binding of a signal to a receptor brings about change in cells is the topic of this section. We will examine a few of the major receptor types and the consequences of signal binding to these receptors. Although the specific molecular components of the various signal transduction pathways differ, they all have some features in common (Figure 7.131):

- The binding of a signal to its receptor is usually followed by the generation of a new signal(s) within the cell. The process by which the original signal is converted to a different form and passed on within the cell to bring about change is called signal transduction.
- Most signaling pathways have multiple signal transduction steps by which the signal is relayed through a series of molecular messengers that can amplify and distribute the message to various parts of the cell.
- The last of these messengers usually interacts with a target protein(s) and changes its activity, often by phosphorylation.
- When a signal sets a particular pathway in motion, it is acting like an ON switch. This means that once the desired result has been obtained, the cell must have a mechanism that acts as an OFF switch.

Understanding this underlying similarity is helpful, because learning the details of the different pathways becomes merely a matter of identifying which molecular component performs a particular function in each individual case. We will consider several different signal transduction pathways, each mediated by a different kind of receptor.

Ligand-gated ion channel receptors

The simplest and fastest of signal pathways is seen in the case of signals whose receptors are gated ion channels (Figure 7.132). Gated ion channels are made up of multiple transmembrane proteins that create a pore, or channel, in the cell membrane. Depending upon its type, each ion channel is specific to the passage of a particular ionic species. The term "gated" refers to the fact that the ion channel is controlled by a "gate" which must be opened to allow the ions through. The gates are opened by the binding of an incoming signal (ligand) to the receptor, allowing the almost instantaneous passage of millions of ions from one side of the membrane to the other. Changes in the interior environment of the cell are thus brought about in microseconds and in a single step.

Swift response

This type of swift response is seen, for example, in neuromuscular junctions, where muscle cells respond to a message from the neighboring nerve cell (Figure 7.133). The nerve cell releases a neurotransmitter signal into the synaptic cleft, which is the space between the nerve cell and the muscle cell it is "talking to". An example of such a neurotransmitter signal is acetylcholine. When the acetylcholine molecules are released into the synaptic cleft, they diffuse rapidly till they reach their receptors on the membrane of the muscle cell. The binding of the acetylcholine to its receptor, an ion channel on the membrane of the muscle cell, causes the gate in the ion channel to open. The resulting ion flow through the channel can immediately change the membrane potential of the cell. This, in turn, can trigger other changes in the cell.

The speed with which changes are brought about in neurotransmitter signaling is evident when you think about how quickly you remove your hand from a hot surface. Sensory neurons carry information to the brain from your hand on the hot surface and motor neurons signal to your muscles to move the hand, in less time than it took you to read this sentence!

Nuclear hormone receptors

The receptors for signals like steroid hormones are part of a large group of proteins known as the nuclear hormone receptor superfamily. These receptors recognize and bind not only steroid hormones, but also retinoic acid, thyroid hormone, vitamin D and other signals. The subset of the nuclear hormone receptors that bind steroid hormones are intracellular proteins. Steroid hormones (Figure 7.135), as you are aware, are related to cholesterol, and as hydrophobic molecules, they are able to cross the cell membrane by themselves. This is unusual, as most signals coming to cells are incapable of crossing the plasma membrane, and thus, must have cell surface receptors.

Once within the cell, steroid hormones bind to their receptors, which may reside in the cytoplasm or in the nucleus. Steroid hormone receptors are proteins with a double life: they are actually dormant transcription regulators that are inactive till a steroid hormone binds and causes a conformational change in them. When this happens, the receptors, with the hormone bound, bind to regulatory sequences in the DNA and modulate gene expression. Because steroid hormone receptors act by modulating gene expression, the responses to steroid hormones are relatively slow. (There are also some effects of steroid hormones that do not involve transcriptional regulation, but the majority work through changing gene expression.) Like other transcriptional activators, steroid receptors have a DNA-binding domain (DBD) and an activation domain. They also have a ligand-binding domain (LBD) that binds the hormone.

Glucocorticoid receptor

Examples of such signaling pathways are those mediated by the glucocorticoid receptor (Figures 7.136 & 7.137). Glucocorticoids, sometimes described as stress hormones, are made and secreted by the adrenal cortex. Physiologically, they serve to maintain homeostasis in the face of stress and exhibit strong anti-inflammatory and immunosuppressive properties. Because of these effects, synthetic glucocorticoids are used in the treatment of a number of diseases from asthma and rheumatoid arthritis to multiple sclerosis. All of these effects are mediated through the signaling pathway which starts with the binding of a glucocorticoid hormone to its receptor. Recall that steroids can cross the plasma membrane, so glucocorticoids can diffuse into the cell and bind their receptors which are in the cytoplasm.

In the absence of the signal, glucocorticoid receptors are found bound to a protein chaperone, Hsp90 (Figure 7.137). This keeps the receptors from being transported to the nucleus. When a glucocorticoid molecule binds the receptor, the receptor undergoes a conformational change and dissociates from the Hsp90. The receptor, then, with the hormone bound, translocates into the nucleus. In the nucleus, it can increase the transcription of target genes by binding to specific regulatory sequences (labeled HRE for

hormone-response elements). The binding of the hormone-receptor complex to the regulatory elements of hormone-responsive genes modulates their expression. Many of these genes encode anti-inflammatory proteins, and their increased production accounts for the physiological effect of corticosteroid therapies.

The steroid receptor pathways are relatively simple and have only a couple of steps (Figure 7.138). Most other signaling pathways involve multiple steps in which the original signal is passed on and amplified through a number of intermediate steps, before the cell responds to the signal.

Cell surface receptors

We will now take a look at two signaling pathways, each mediated by a major class of cell surface receptor- the G-protein coupled receptors (GPCRs) and the receptor tyrosine kinases (RTKs). While the specific details of the signaling pathways that follow the binding of signals to each of these receptor types are different, it is easier to learn them when you can see what the pathways have in common, namely, interaction of the signal with a receptor, followed by relaying and amplification of the signal through a variable number of intermediate molecules, with the last of these molecules interacting with a target or target proteins and modifying their activity in the cell.

G-protein coupled receptors

G-protein coupled receptors (GPCRs) are involved in responses of cells to many different kinds of signals, from epinephrine, to odors, to light. In fact, a variety of physiological phenomena including vision, taste, smell, and the fight-or-flight response are mediated by GPCRs. What are G-protein coupled receptors?

G-protein coupled receptors are cell surface receptors that pass on the signals that they receive with the help of guanine nucleotide binding proteins (a.k.a. G-proteins). Before thinking any further about the signaling pathways downstream of GPCRs, it is necessary to know a few important facts about these receptors and the G-proteins that assist them.

Though there are hundreds of different G-protein coupled receptors, they all have the same basic structure (Figure 7.139):

They all consist of a single polypeptide chain that threads back and forth seven times through the lipid bilayer of the plasma membrane. For this reason, they are sometimes called seven-pass transmembrane (7TM) receptors. One end of the polypeptide forms the extracellular domain that binds the signal while the other end is in the cytosol of the cell.

When a ligand (signal) binds the extracellular domain of a GPCR, the receptor undergoes a conformational change, on its cytoplasmic side, that allows it to interact with a G-protein that will then pass the signal on to other intermediates in the signaling pathway.

G-proteins

What is a G-protein? As noted above, a G-protein is a guanine nucleotide-binding protein that can interact with a G-protein linked receptor. G-proteins are associated with the cytosolic side of the plasma membrane, where they are ideally situated to interact with the tail of the GPCR, when a signal binds to the GPCR. There are many different G-proteins, all of which share a characteristic structure- they are composed of three subunits called α, β and γ (Figure 7.140). Because of this, they are sometimes called heterotrimeric G proteins (hetero=different, trimeric= having three parts).

Ligand binding

The guanine nucleotide binding site is on the α subunit of the G-protein. This site can bind GDP or GTP. The α subunit also has a GTPase activity, i.e., it is capable of hydrolyzing a GTP molecule bound to it into GDP.

In the unstimulated state of the cell, that is, in the absence of a signal bound to the GPCR, the G-proteins are found in the trimeric form (α-β-γ bound together) and the α subunit has a GDP molecule bound to it. In this form, the α subunit is inactive. With this background on the structure and general properties of the GPCRs and the G-proteins, we can now look at what happens when a signal arrives at the cell surface and binds to a GPCR (Figure 7.141).

The signaling pathway

The binding of a signal molecule by the extracellular part of the G-protein linked receptor causes the cytosolic tail of the receptor to interact with, and alter the conformation of, a G-protein associated with the inner face of the plasma membrane.

This has two consequences. First, the α subunit of the G-protein loses its GDP and binds a GTP, instead. Second, the G-protein breaks up into the GTP-bound α part and the β-γ part.

The binding of GTP to the α subunit and its dissociation from the β-γ subunits activate the α subunit. The activated α subunit can diffuse freely along the cytosolic face of the plasma membrane and act upon its targets. (The β-γ unit is also capable of activating its own targets.)

What happens when G-proteins interact with their target proteins? That depends on what the target is. G-proteins interact with different kinds of target proteins, of which we will examine two major categories:

Ion channels

We have earlier seen that some gated ion channels can be opened or closed by the direct binding of neurotransmitters to a receptor that is an ion-channel protein. In other cases, ion channels are regulated by the binding of G-proteins. That is, instead of the signal directly binding to the ion channel, it binds to a GPCR, which activates a G-protein that then may cause opening of the ion channel, either directly, by binding to the channel, or indirectly, through activating other proteins that can bind to the channel. The change in the distribution of ions across the plasma membrane causes a change in the membrane potential.

Enzyme activation

The interaction of G-proteins with their target enzymes can regulate the activity of the enzyme, either increasing or decreasing its activity. The change in activity of the target enzyme, in turn, results in downstream changes in other proteins in the cell, and alters the metabolic state of the cell. This is best understood by examining the well-studied response of cells to epinephrine, mediated through the β-adrenergic receptor, a type of G-protein coupled receptor.

Epinephrine (Figure 7.142), also known as adrenaline, is a catecholamine that plays an important role in the body's 'fight or flight' response. In response to stressful stimuli, epinephrine is secreted into the blood, to be carried to target organs whose cells will respond to this signal. If you were walking down a dark alley in an iffy neighborhood, and you heard footsteps behind you, your brain would respond to potential danger by sending signals that ultimately cause the adrenal cortex to secrete epinephrine into the blood stream. The epinephrine circulating in your system has many effects, including increasing your heart rate, but among its prime targets are your muscle cells. The reason for this is that your muscle cells store energy in the form of glycogen, a polymer of glucose. If you need to run or fight off an assailant, your cells will need energy in the form of glucose.

But how does epinephrine get your cells to break down the glycogen into glucose? Binding of epinephrine to the β-adrenergic receptor on the surface of the cells causes the receptor to activate a G-protein associated with its cytoplasmic tail. As described above, this leads to the α subunit exchanging its GDP for GTP and dissociating from the β-γ subunits. The activated α subunit then interacts with the enzyme adenylate cyclase (also known as adenylyl cyclase) stimulating it to produce cyclic AMP (cAMP) from ATP. Cyclic AMP is often described as a "second messenger", in that it serves to spread the signal received by the cell. How does cAMP accomplish this?

cAMP molecules bind to, and activate an enzyme, protein kinase A (PKA - Figure 7.145). PKA is composed of two catalytic and two regulatory subunits that are bound tightly together. Upon binding of cAMP, the catalytic subunits are released from the regulatory subunits, allowing the enzyme to carry out its function, namely phosphorylating other proteins. Thus, cAMP can regulate the activity of PKA, which in turn, by phosphorylating other proteins can change their activity. In this case, the relevant protein that is activated is an enzyme, phosphorylase kinase. This enzyme can then phosphorylate and activate glycogen phosphorylase, the enzyme ultimately responsible for breaking glycogen down into glucose-1-phosphate - readily converted to glucose. The activation of glycogen phosphorylase supplies the cells with the glucose they need, allowing you to fight or flee, as you might see fit. Simultaneously, PKA also phosphorylates another enzyme, glycogen synthase. In the case of glycogen synthase, phosphorylation inactivates it, and prevents free glucose from being used up for glycogen synthesis, ensuring that your cells are amply supplied with glucose (Figure 7.146).

Common pattern

Although the steps described above seem complicated, they follow the simple pattern outlined at the beginning of this section:
• Binding of signal to receptor
• Several steps where the signal is passed on through intermediate molecules (G-proteins, adenylate cyclase, cAMP, and finally,

PKA)

• Phosphorylation of target proteins by the kinase, leading to changes in the cell. The specific changes depend on the proteins that are phosphorylated by the PKA.

Why so many steps? If you need to activate glycogen phosphorylase to break down glucose in a hurry, why not have a system in which binding of a signal to the receptor directly activated the target enzyme?

The answer to this puzzle is simple: there is amplification of the signal at every step of the pathway. A single signal molecule binding to a receptor sets in motion a cascade of reactions, with the signal getting larger at each step, so that binding of one epinephrine molecule to its receptor results in the activation of a million glycogen phosphorylase enzyme molecules!

Turning signals off

If the signal binding to the receptor serves as a switch that sets these events in motion, there must be mechanisms to turn the pathway off. The first is at the level of the receptor itself. A kinase called G-protein receptor kinase (GRK) phosphorylates the cytoplasmic tail of the receptor. The phosphorylated tail is then bound by a protein called arrestin, preventing further interaction with a G-protein.

The next point of control is at the G-protein. Recall that the α subunit of the G-protein is in its free and activated state when it has GTP bound, and that it associates with the β-γ subunits and has a GDP bound when it is inactive. We also know that the α subunit has an activity that enables it to hydrolyze GTP to GDP. This GTP-hydrolyzing activity makes it possible for the α subunit, once it has completed its task, to return to its GDP bound state, re-associate with the β-γ part and become inactive again.

A third "off switch" is further down the signaling pathway, and controls the level of cAMP. We just noted that cAMP levels increase when adenylate cyclase is activated. When its job is done, cAMP is broken down by an enzyme called phosphodiesterase (Figure 7.147). When cAMP levels drop, PKA returns to its inactive state, putting a halt to the changes brought about by the activation of adenylate cyclase by an activated G-protein.

Yet another way that the effects of this pathway can be turned off is at the level of the phosphorylated target proteins. These proteins, which are activated by phosphorylation, can be returned to their inactive state by the removal of the phosphates by phosphatases.

Receptor tyrosine kinases

Another major class of cell surface receptors are the receptor tyrosine kinases or RTKs. Like the GPCRs, receptor tyrosine kinases bind a signal, then pass the message on through a series of intracellular molecules, the last of which acts on target proteins to change the state of the cell.

As the name suggests, a receptor tyrosine kinase is a cell surface receptor that also has a tyrosine kinase activity. The signal binding domain of the receptor tyrosine kinase is on the cell surface, while the tyrosine kinase enzymatic activity resides in the cytoplasmic part of the protein (Figure 7.148). A transmembrane α helix connects these two regions of the receptor.

What happens when signal molecules bind to receptor tyrosine kinases? Binding of signal molecules to the extracellular domains of receptor tyrosine kinase proteins causes two receptor molecules to dimerize (come together and associate - Figure 7.149). This brings the cytoplasmic tails of the receptors close to each other and causes the tyrosine kinase activity of these tails to be turned on. The activated tails then phosphorylate each other on several tyrosine residues (Figure 7.150). This is called autophosphorylation.

The phosphorylation of tyrosines on the receptor tails triggers the assembly of an intracellular signaling complex on the tails. The newly phosphorylated tyrosines serve as binding sites for a variety of signaling proteins that then pass the message on to yet other proteins to bring about changes in the cell. Receptor tyrosine kinases mediate responses to a large number of signals, including peptide hormones like insulin and growth factors like epidermal growth factor (EGF). We will examine how insulin and EGF act on cells by binding to receptor tyrosine kinases.

Insulin receptor

Insulin plays a central role in the uptake of glucose from the bloodstream. It increases glucose uptake by stimulating the movement of glucose receptor GLUT4 to the plasma membrane of cells.

How does insulin increase GLUT4 concentrations in the cell membrane? The binding of insulin to the insulin receptor (IR - Figure 7.151), results in dimerization of the receptor monomers and subsequent autophosphorylation of the cytosolic kinase domains. The activated tyrosine kinase domains also phosphorylate intracellular proteins called Insulin Receptor Substrates or IRS proteins. These proteins interact with, and activate another kinase called the PI3-kinase. PI3-kinase then catalyzes the formation of the lipid molecule PIP3, which serves to activate yet another kinase, PDK1, which in turn, activates the Akt group of kinases. It is this group of enzymes that appears to increase the translocation of the GLUT4 to the plasma membrane (Figure 7.152), as cells that lack functional Akts exhibit poor glucose uptake and insulin resistance.

EGFR pathway

Epidermal growth factor, EGF, is an important signaling molecule involved in growth, proliferation and differentiation in mammalian cells. EGF acts through the EGF receptor, EGFR, a receptor tyrosine kinase (Figure 7.153). Because of its role in stimulating cell proliferation and because overexpression of EGFR is associated with some kinds of cancers, EGFR is the target for many anti-cancer therapies. We can trace the signal transduction pathway from the binding of EGF to its receptor to the stimulation of cell division.

EGF binding to the EGFR is followed by receptor dimerization and stimulation of the tyrosine kinase activity of the cytosolic domains of the EGFR. Autophosphorylation of the receptor tails is followed by the assembly of a signaling complex nucleated by the binding of proteins that recognize phosphotyrosine residues. An important protein that is subsequently activated by the signaling complexes on the receptor tyrosine kinases is called Ras (Figure 7.154). The Ras protein is a monomeric guanine nucleotide binding protein that is associated with the cytosolic face of the plasma membrane
(in fact, it is a lot like the α subunit of trimeric G-proteins). Just like the α subunit of a G-protein, Ras is active when GTP is bound to it and inactive when GDP is bound to it. Also, like the α subunit, Ras can hydrolyze the GTP to GDP.

Ras activation

Activation of Ras accompanies the exchange of the GDP bound to the inactive Ras for a GTP. Activated Ras triggers a phosphorylation cascade of three protein kinases, which relay and distribute the signal. These protein kinases are members of a group called the MAP kinases (Mitogen Activated Protein Kinases). The final kinase in this cascade phosphorylates various target proteins, including enzymes and transcriptional activators that regulate gene expression.

The phosphorylation of various enzymes can alter their activities, and set off new chemical reactions in the cell, while the phosphorylation of transcriptional activators can change which genes are expressed. The combined effect of changes in gene expression and protein activity alter the cell's physiological state and promote cell division.

Once again, in following the path of signal transduction mediated by RTKs, it is possible to discern the same basic pattern of events: a signal is bound by the extracellular domains of receptor tyrosine kinases, resulting in receptor dimerization and autophosphorylation of the cytosolic tails, thus conveying the message to the interior of the cell.

The message is then passed on via a signaling complex to proteins that stimulate a series of kinases. The terminal kinase in the cascade acts on target proteins and brings about in changes in protein activities.

What is the OFF switch for RTKs? It turns out that RTKs with the signal bound can be endocytosed into the cell and broken down. That is, the region of the plasma membrane that the RTK is on can be internally pinched off into a vesicle containing the ligand-bound receptor which is then targeted for degradation.

Ras, which is activated by GTP binding, can also be deactivated by hydrolysis of the GTP to GDP. The importance of this mechanism for shutting down the pathway is evident in cells that have a mutant ras gene encoding a Ras protein with defective GTPase activity. Unable to shut off Ras, the cells continue to receive a signal to proliferate. The National Cancer Institute estimates that more than 30% of human cancers are driven by mutations in ras genes.

The descriptions above provide a very simple sketch of some of the major classes of receptors and deal primarily with the mechanistic details of the steps by which signals received by various types of receptors bring about changes in cells. A major take-home lesson is the essential similarity of the different pathways. Another point to keep in mind is that while we have looked at each individual pathway in isolation, a cell, at any given time receives multiple signals that set off a variety of different responses at once (Figure 7.155). The pathways described above show a considerable degree of "cross-talk" and the response to any given signal

is affected by the other signals that the cell receives simultaneously. The multitude of different receptors, signals, and the combinations thereof are the means by which cells are able to respond to an enormous variety of different circumstances.

RTKs, cancer and cancer therapies

As described above, binding of EGF to its receptor triggers a signaling pathway that results in the activation of a series of Mitogen Activated Protein Kinases (MAP kinases). These kinases are so-called because they are activated by a mitogen, a molecule, like EGF and other growth factors, that stimulates mitosis or cell division. The final kinase in the MAP kinase cascade phosphorylates a number of target proteins, many of them transcription factors, that when activated, increase the expression of genes associated with cell proliferation.

Given that the EGF-receptor pathway normally functions to stimulate cell division, it is not surprising that malfunctions in the pathway could lead to uncontrolled cell proliferation, or cancer. Next, we will take a brief look at some examples of such defects.

HER2

The human EGF receptor (HER) family has four members, HER1, HER2, HER3 and HER4. These are all receptor tyrosine kinases, cell surface receptors that bind EGF (Figure 7.157) and stimulate cell proliferation.

A crucial step in the signal transduction pathway is the dimerization of the receptors following binding of the signal, EGF, to the receptor. While HER1, HER3 and HER4 must bind the signal to dimerize, the structure of the HER2 receptor can, apparently, allow the receptor monomers to dimerize independently of EGF binding.

This means that the downstream events of the signaling pathway can be triggered even in the absence of a growth signal. In normal cells, only a few HER2 receptors are expressed at the cell surface, so this property of HER2 plays a relatively minor role in stimulating cell division. However, in about a quarter of breast cancer patients, HER2 receptors are overexpressed, leading to increased dimerization and subsequent uncontrolled cell proliferation.

Breast cancers that are HER2-positive can be more aggressive with a greater tendency to metastasize (spread) so therapy that blocks HER2 signaling is key in successful treatment of such cancers. Herceptin, a monoclonal antibody against the HER2 receptor, has been shown to be an effective treatment against Her2-positive breast cancers. Herceptin works by binding specifically to the extracellular domain of the HER2 receptor (Figure 7.158). This prevents dimerization of the receptor and thus blocks downstream signaling. Additionally, the binding of the Herceptin antibody to the receptor signals the immune system to destroy the HER2-positive cells.

Bcr-abl

Another example of a cancer caused by defects in an RTK signaling pathway is chronic myeloid leukemia (CML). Patients with CML have an abnormal receptor tyrosine kinase that is the product of a hybrid gene called bcr-abl, formed by the breakage and rejoining of chromosomes 9 and 22. This abnormal tyrosine kinase is constitutively dimerized, even when no signal is bound. As a result, it continuously signals cells to divide, leading to the massive proliferation of a type of blood cells called granulocytes.

As with HER2, the problem in CML is a receptor tyrosine kinase that dimerizes in the absence of a growth signal. The approach in this case was to target the next step in the signaling pathway. As you know, dimerization of RTKs activates the tyrosine kinase domain of the receptor, which results in the autophosphorylation of the cytoplasmic domains of both monomers. The phosphorylated tyrosines serve to recruit a number of other signaling proteins that pass the signal on within the cell.

In the case of the bcr-abl RTK, the drug Gleevec (imatinib) was designed to bind near the ATP-binding site of the tyrosine kinase domain. This "locks" the site in a conformation that inhibits the enzymatic activity of the tyrosine kinase and thus blocks downstream signaling. With no "grow" signal passed on, cells stop proliferating.

Information Processing: Signaling

827

YouTube Lectures

by Kevin

HERE & HERE

828

Figure 7.130 - Schematic representation of a transmembrane receptor protein. E = extracellular; P = plasma membrane; I = intracellular

Wikipedia

Figure 7.129 - Some examples of signal molecules

829

Figure 7.132 - Ligand-gated ion channel receptor opening in response to a signal (ligand)

Wikipedia

Figure 7.131 -General features of signal transduction pathways

830

Figure 7.133 - Neuromuscular signaling - A = motor neuron axon; B = axon terminal; C = synaptic cleft; D = muscle cell; E = myofibril . Steps in the process - 1) action potential reaches the axon terminal; 2) voltage-dependent calcium gates open; (3) neurotransmitter vesicles fuse with the presynaptic membrane and acetylcholine (ACh) released into the synaptic cleft; (4) ACh binds to postsynaptic receptors on the sarcolemma; (5) ACh binding causes ion channels to open and allows sodium ions to flow across the membrane into the muscle cell; 6) flow of sodium ions across the membrane into the muscle cell generates action potential which travels to the myofibril and results in muscle contraction.

Wikipedia

831

Figure 7.134 - Nerve systems

Wikipedia

832

Figure 7.135 - Steroid hormones structures, with the names of their receptors

Wikipedia

YouTube Lectures

by Kevin

HERE & HERE

833

Figure 7.136 - Glucocorticoid receptor with its three domains - DNA binding (left), activator domain (top), and ligand binding domain (boxed).

Wikipedia

Figure 7.137 - Glucocorticoid signaling pathway

Wikipedia

834

Figure 7.138 - Steroid hormone signaling

Image by Aleia Kim

835

Figure 7.139 - Structure of a G-protein linked receptor

Wikipedia

836

Figure 7.140- A heterotrimeric G-protein: α subunit in blue, βγ subunits red and green

Interactive Learning

Module

HERE

837

Figure 7.141 - Cycle of G-protein activation - 1) binding of ligand; 2) change of receptor structure; 3) stimulation of α-subunit; 4) binding of GTP, release of GDP; 5) separation of α-subunit from β-γ; 6) hydrolysis of GTP by α-subunit and return to inactive state.

Wikipedia

YouTube Lectures

by Kevin

HERE & HERE

838

Figure 7.142 - β2-adrenergic receptor embedded in membrane (gray)

Wikipedia

Figure 7.143 - Epinephrine

Wikipedia

839

Figure 7.144 - G-protein coupled receptor. Signal starts with ligand binding (orange circle). Gs = G-protein; AC = adenylate cyclase.

Wikipedia

Figure 7.145 - Activation of Protein Kinase A by cAMP

Image by Martha Baker

840

Figure 7.146 - Simultaneous activation of glycogen breakdown and inhibition of glycogen synthesis by epinephrine's binding of b-adrenergic receptor. Red enzyme names = activated forms; black enzyme names = inactivated forms; GPb = glycogen phosphorylase b; GPa = glycogen phosphorylase a.

Image by Penelope Irving

841

β-Adrenergic Signaling Off Switches

1. GRK Phosphorylates Receptor Tail

Receptor Tail Bound by Arrestin

2. α Subunit G-protein Cleaves GTP to GDP

β-γ subunits Reassociate with α Subunit

3. cAMP Hydrolyzed by Phosphodiesterase

PKA Becomes Inactive

4. Dephosphorylation of Phosphorylated Proteins by Phosphoprotein Phosphatase

β-Adrenergic Signaling On Switches

1. Binding of Signal Molecule to Receptor

2. Passage of Signal Through Several Molecules (G-proteins, Adenylate Cyclase, cAMP, PKA)

3. Phosphorylation of Target Proteins

842

Figure 7.147 - Cyclic AMP is broken down by phosphodiesterase

Figure 7.148 - Structure of a receptor tyrosine kinase

YouTube Lectures

by Kevin

HERE & HERE

843

Figure 7.149 - Signal binding results in receptor dimerization and activation of tyrosine kinase activity

Figure 7.150 - Activated tyrosine kinases phosphorylate tyrosines on the receptor tails.

Figure 7.151 -The insulin receptor, a receptor tyrosine kinase

Wikipedia

Figure 7.152 - Effects of insulin binding to its receptor tyrosine kinase: 1) insulin binding; 2) activation of protein activation cascades. These include: 3) translocation of Glut-4 transporter to plasma membrane and influx of glucose; 4) glycogen synthesis; 5) glycolysis; and 6) fatty acid synthesis.

Wikipedia

844

Interactive Learning

Module

HERE

845

Figure 7.153 - EGFR signaling beginning at top with binding of EGF, dimerization of receptor, transmission of signal through proteins, activation of kinases, phosphorylation of transcription factors and effects on transcription

Image by Aleia Kim

Figure 7.154 - Ras with GTP bound

Wikipedia

This page titled 7.1: Signaling is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **7.9: Signaling** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

SECTION OVERVIEW

7.2: How Cells Obtain Energy

7.2.1: Energy and Metabolism

7.2.2: Glycolysis

7.2.3: Citric Acid Cycle and Oxidative Phosphorylation

7.2.4: Fermentation

7.2.5: Connections to Other Metabolic Pathways

7.2.E: How Cells Obtain Energy (Exercises)

This page titled 7.2: How Cells Obtain Energy is shared under a CC BY license and was authored, remixed, and/or curated by .

7.2.1: Energy and Metabolism

Scientists use the term bioenergetics to describe the concept of energy flow (Figure **7.2.1.1**) through living systems, such as cells. Cellular processes such as the building and breaking down of complex molecules occur through stepwise chemical reactions. Some of these chemical reactions are spontaneous and release energy, whereas others require energy to proceed. Just as living things must continually consume food to replenish their energy supplies, cells must continually produce more energy to replenish that used by the many energy-requiring chemical reactions that constantly take place. Together, all of the chemical reactions that take place inside cells, including those that consume or generate energy, are referred to as the cell's metabolism.

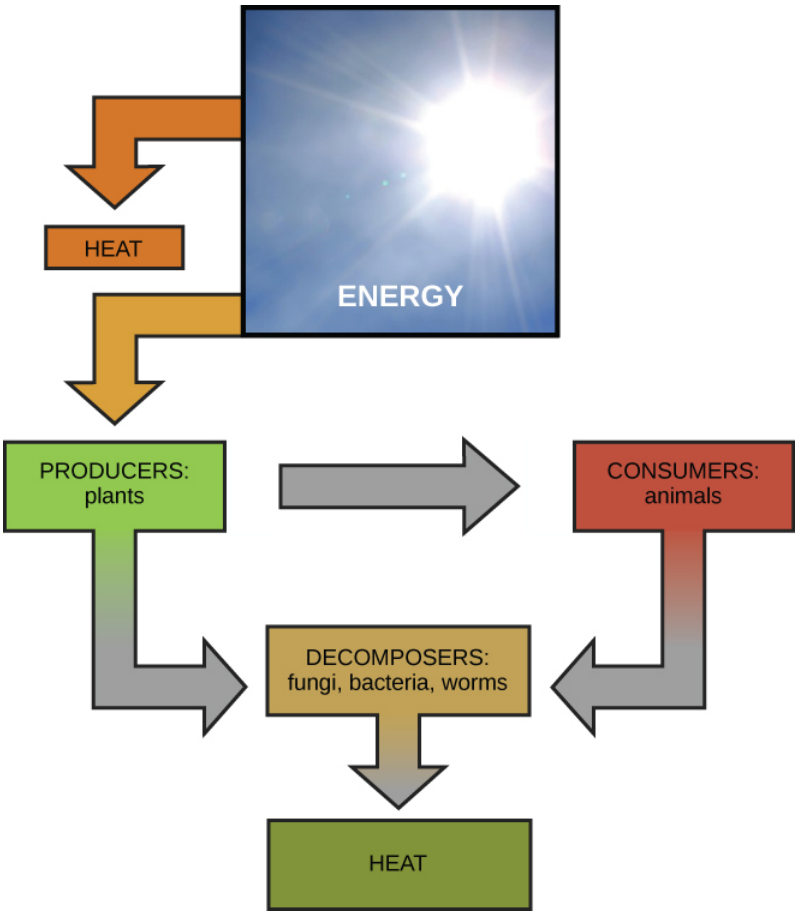


Figure 7.2.1.1: Ultimately, most life forms get their energy from the sun. Plants use photosynthesis to capture sunlight, and herbivores eat the plants to obtain energy. Carnivores eat the herbivores, and eventual decomposition of plant and animal material contributes to the nutrient pool.

Metabolic Pathways

Consider the metabolism of sugar. This is a classic example of one of the many cellular processes that use and produce energy. Living things consume sugars as a major energy source, because sugar molecules have a great deal of energy stored within their bonds. For the most part, photosynthesizing organisms like plants produce these sugars. During photosynthesis, plants use energy (originally from sunlight) to convert carbon dioxide gas (CO_2) into sugar molecules (like glucose: $C_6H_{12}O_6$). They consume carbon dioxide and produce oxygen as a waste product. This reaction is summarized as:

$$6\,CO_2 + 6\,H_2O \longrightarrow C_6H_{12}O_6 + 6\,O_2$$

Because this process involves synthesizing an energy-storing molecule, it requires energy input to proceed. During the light reactions of photosynthesis, energy is provided by a molecule called adenosine triphosphate (ATP), which is the primary energy currency of all cells. Just as the dollar is used as currency to buy goods, cells use molecules of ATP as energy currency to perform immediate work. In contrast, energy-storage molecules such as glucose are consumed only to be broken down to use their energy. The reaction that harvests the energy of a sugar molecule in cells requiring oxygen to survive can be summarized by the reverse

reaction to photosynthesis. In this reaction, oxygen is consumed and carbon dioxide is released as a waste product. The reaction is summarized as:

$$C_6H_{12}O_6 + 6\,O_2 \longrightarrow 6\,H_2O + 6\,CO_2$$

Both of these reactions involve many steps.

The processes of making and breaking down sugar molecules illustrate two examples of metabolic pathways. A metabolic pathway is a series of chemical reactions that takes a starting molecule and modifies it, step-by-step, through a series of metabolic intermediates, eventually yielding a final product. In the example of sugar metabolism, the first metabolic pathway synthesized sugar from smaller molecules, and the other pathway broke sugar down into smaller molecules. These two opposite processes—the first requiring energy and the second producing energy—are referred to as anabolic pathways (building polymers) and catabolic pathways (breaking down polymers into their monomers), respectively. Consequently, metabolism is composed of synthesis (anabolism) and degradation (catabolism) (Figure **7.2.1.2**).

It is important to know that the chemical reactions of metabolic pathways do not take place on their own. Each reaction step is facilitated, or catalyzed, by a protein called an enzyme. Enzymes are important for catalyzing all types of biological reactions—those that require energy as well as those that release energy.

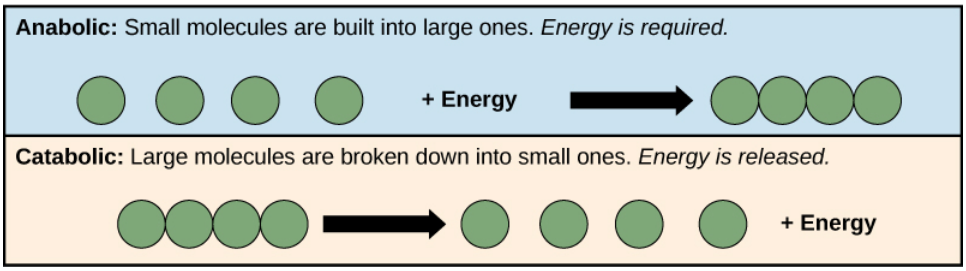


Figure 7.2.1.2: Catabolic pathways are those that generate energy by breaking down larger molecules. Anabolic pathways are those that require energy to synthesize larger molecules. Both types of pathways are required for maintaining the cell's energy balance.

Energy

Thermodynamics refers to the study of energy and energy transfer involving physical matter. The matter relevant to a particular case of energy transfer is called a system, and everything outside of that matter is called the surroundings. For instance, when heating a pot of water on the stove, the system includes the stove, the pot, and the water. Energy is transferred within the system (between the stove, pot, and water). There are two types of systems: open and closed. In an open system, energy can be exchanged with its surroundings. The stovetop system is open because heat can be lost to the air. A closed system cannot exchange energy with its surroundings.

Biological organisms are open systems. Energy is exchanged between them and their surroundings as they use energy from the sun to perform photosynthesis or consume energy-storing molecules and release energy to the environment by doing work and releasing heat. Like all things in the physical world, energy is subject to physical laws. The laws of thermodynamics govern the transfer of energy in and among all systems in the universe.

In general, energy is defined as the ability to do work, or to create some kind of change. Energy exists in different forms. For example, electrical energy, light energy, and heat energy are all different types of energy. To appreciate the way energy flows into and out of biological systems, it is important to understand two of the physical laws that govern energy.

Thermodynamics

The first law of thermodynamics states that the total amount of energy in the universe is constant and conserved. In other words, there has always been, and always will be, exactly the same amount of energy in the universe. Energy exists in many different forms. According to the first law of thermodynamics, energy may be transferred from place to place or transformed into different forms, but it cannot be created or destroyed. The transfers and transformations of energy take place around us all the time. Light bulbs transform electrical energy into light and heat energy. Gas stoves transform chemical energy from natural gas into heat

energy. Plants perform one of the most biologically useful energy transformations on earth: that of converting the energy of sunlight to chemical energy stored within organic molecules (Figure **7.2.1.1**). Some examples of energy transformations are shown in Figure **7.2.1.3**.

The challenge for all living organisms is to obtain energy from their surroundings in forms that they can transfer or transform into usable energy to do work. Living cells have evolved to meet this challenge. Chemical energy stored within organic molecules such as sugars and fats is transferred and transformed through a series of cellular chemical reactions into energy within molecules of ATP. Energy in ATP molecules is easily accessible to do work. Examples of the types of work that cells need to do include building complex molecules, transporting materials, powering the motion of cilia or flagella, and contracting muscle fibers to create movement.

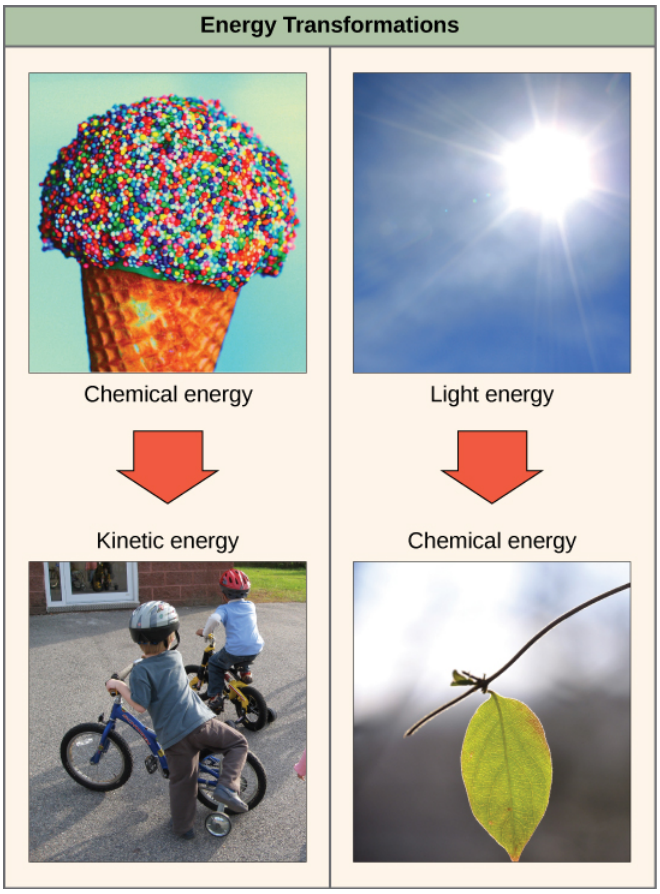


Figure 7.2.1.3: Shown are some examples of energy transferred and transformed from one system to another and from one form to another. The food we consume provides our cells with the energy required to carry out bodily functions, just as light energy provides plants with the means to create the chemical energy they need. (credit "ice cream": modification of work by D. Sharon Pruitt; credit "kids": modification of work by Max from Providence; credit "leaf": modification of work by Cory Zanker)

A living cell's primary tasks of obtaining, transforming, and using energy to do work may seem simple. However, the second law of thermodynamics explains why these tasks are harder than they appear. All energy transfers and transformations are never completely efficient. In every energy transfer, some amount of energy is lost in a form that is unusable. In most cases, this form is heat energy. Thermodynamically, heat energy is defined as the energy transferred from one system to another that is not work. For example, when a light bulb is turned on, some of the energy being converted from electrical energy into light energy is lost as heat energy. Likewise, some energy is lost as heat energy during cellular metabolic reactions.

An important concept in physical systems is that of order and disorder. The more energy that is lost by a system to its surroundings, the less ordered and more random the system is. Scientists refer to the measure of randomness or disorder within a system as entropy. High entropy means high disorder and low energy. Molecules and chemical reactions have varying entropy as well. For example, entropy increases as molecules at a high concentration in one place diffuse and spread out. The second law of thermodynamics says that energy will always be lost as heat in energy transfers or transformations.

Living things are highly ordered, requiring constant energy input to be maintained in a state of low entropy.

Potential and Kinetic Energy

When an object is in motion, there is energy associated with that object. Think of a wrecking ball. Even a slow-moving wrecking ball can do a great deal of damage to other objects. Energy associated with objects in motion is called kinetic energy (Figure **7.2.1.4**). A speeding bullet, a walking person, and the rapid movement of molecules in the air (which produces heat) all have kinetic energy.

Now what if that same motionless wrecking ball is lifted two stories above ground with a crane? If the suspended wrecking ball is unmoving, is there energy associated with it? The answer is yes. The energy that was required to lift the wrecking ball did not disappear, but is now stored in the wrecking ball by virtue of its position and the force of gravity acting on it. This type of energy is called potential energy (Figure **7.2.1.4**). If the ball were to fall, the potential energy would be transformed into kinetic energy until all of the potential energy was exhausted when the ball rested on the ground. Wrecking balls also swing like a pendulum; through the swing, there is a constant change of potential energy (highest at the top of the swing) to kinetic energy (highest at the bottom of the swing). Other examples of potential energy include the energy of water held behind a dam or a person about to skydive out of an airplane.



Figure 7.2.1.4: Still water has potential energy; moving water, such as in a waterfall or a rapidly flowing river, has kinetic energy. (credit "dam": modification of work by "Pascal"/Flickr; credit "waterfall": modification of work by Frank Gualtieri)

Potential energy is not only associated with the location of matter, but also with the structure of matter. Even a spring on the ground has potential energy if it is compressed; so does a rubber band that is pulled taut. On a molecular level, the bonds that hold the atoms of molecules together exist in a particular structure that has potential energy. Remember that anabolic cellular pathways require energy to synthesize complex molecules from simpler ones and catabolic pathways release energy when complex molecules are broken down. The fact that energy can be released by the breakdown of certain chemical bonds implies that those bonds have potential energy. In fact, there is potential energy stored within the bonds of all the food molecules we eat, which is eventually harnessed for use. This is because these bonds can release energy when broken. The type of potential energy that exists within chemical bonds, and is released when those bonds are broken, is called chemical energy. Chemical energy is responsible for providing living cells with energy from food. The release of energy occurs when the molecular bonds within food molecules are broken.

CONCEPT IN ACTION

Visit the site and select “Pendulum” from the “Work and Energy” menu to see the shifting kinetic and potential energy of a pendulum in motion.

Free and Activation Energy

After learning that chemical reactions release energy when energy-storing bonds are broken, an important next question is the following: How is the energy associated with these chemical reactions quantified and expressed? How can the energy released from one reaction be compared to that of another reaction? A measurement of free energy is used to quantify these energy transfers.

Recall that according to the second law of thermodynamics, all energy transfers involve the loss of some amount of energy in an unusable form such as heat. Free energy specifically refers to the energy associated with a chemical reaction that is available after the losses are accounted for. In other words, free energy is usable energy, or energy that is available to do work.

If energy is released during a chemical reaction, then the change in free energy, signified as ΔG (delta G) will be a negative number. A negative change in free energy also means that the products of the reaction have less free energy than the reactants, because they release some free energy during the reaction. Reactions that have a negative change in free energy and consequently release free energy are called exergonic reactions. Think: *ex*ergonic means energy is *ex*iting the system. These reactions are also referred to as spontaneous reactions, and their products have less stored energy than the reactants. An important distinction must be drawn between the term spontaneous and the idea of a chemical reaction occurring immediately. Contrary to the everyday use of the term, a spontaneous reaction is not one that suddenly or quickly occurs. The rusting of iron is an example of a spontaneous reaction that occurs slowly, little by little, over time.

If a chemical reaction absorbs energy rather than releases energy on balance, then the ΔG for that reaction will be a positive value. In this case, the products have more free energy than the reactants. Thus, the products of these reactions can be thought of as energy-storing molecules. These chemical reactions are called endergonic reactions and they are non-spontaneous. An endergonic reaction will not take place on its own without the addition of free energy.

ART CONNECTION

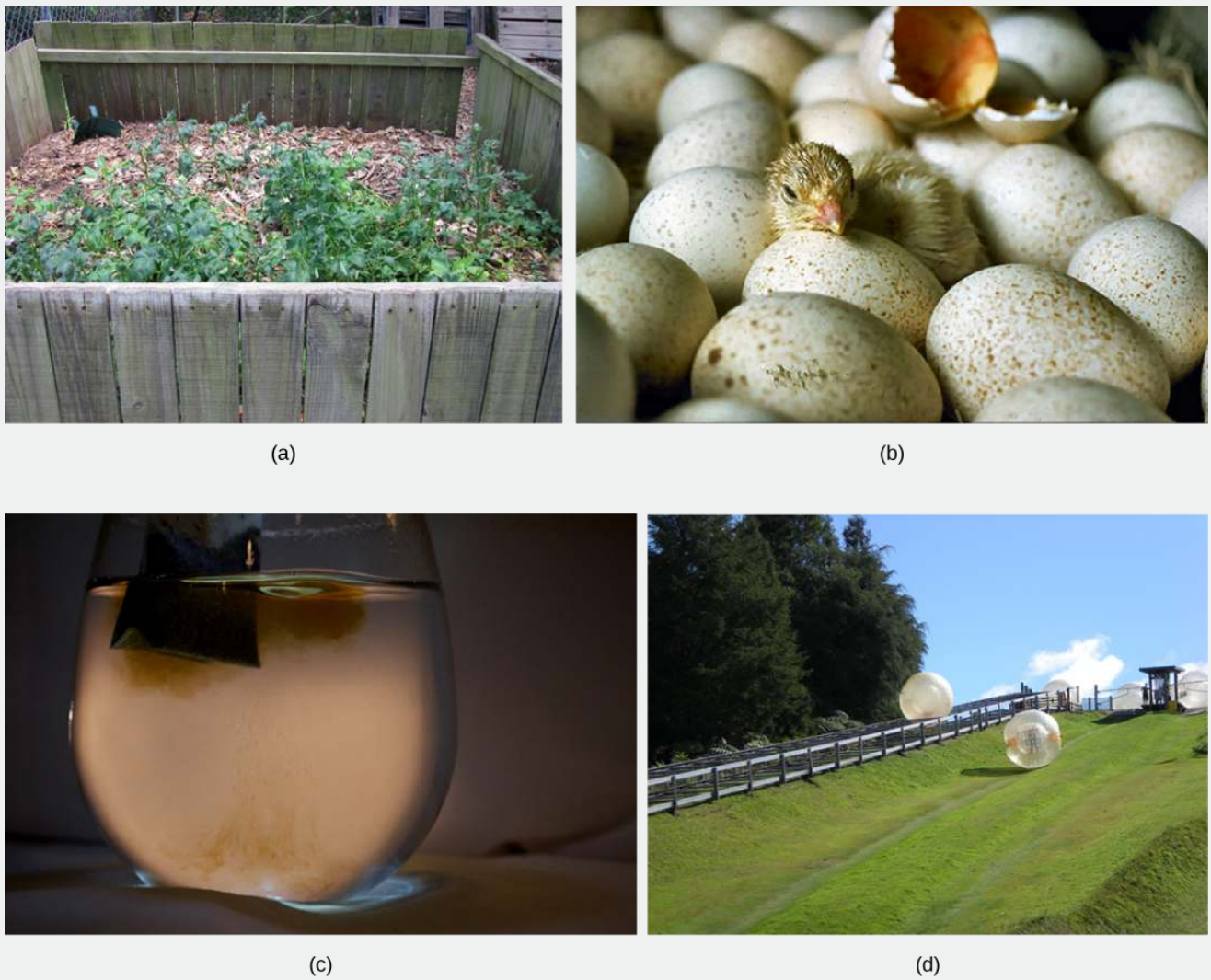
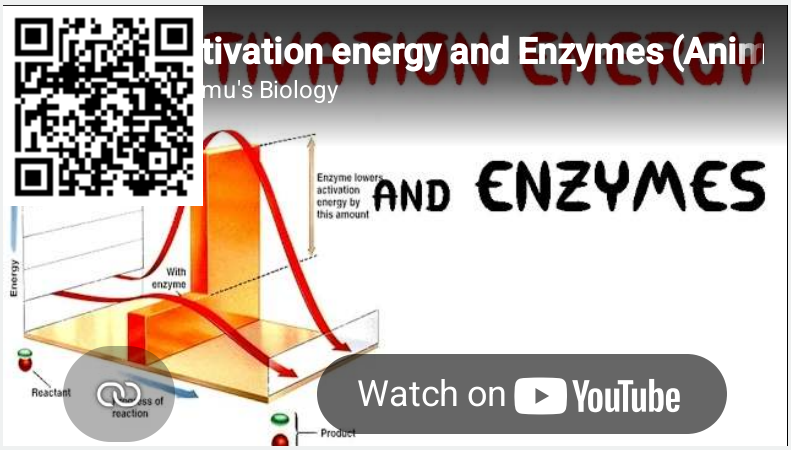


Figure 7.2.1.5: Shown are some examples of endergonic processes (ones that require energy) and exergonic processes (ones that release energy). (credit a: modification of work by Natalie Maynor; credit b: modification of work by USDA; credit c: modification of work by Cory Zanker; credit d: modification of work by Harry Malsch)

Look at each of the processes shown and decide if it is endergonic or exergonic.

There is another important concept that must be considered regarding endergonic and exergonic reactions. Exergonic reactions require a small amount of energy input to get going, before they can proceed with their energy-releasing steps. These reactions have a net release of energy, but still require some energy input in the beginning. This small amount of energy input necessary for all chemical reactions to occur is called the activation energy.

CONCEPT IN ACTION



Watch an animation of the move from free energy to transition state of the reaction.

Enzymes

A substance that helps a chemical reaction to occur is called a catalyst, and the molecules that catalyze biochemical reactions are called enzymes. Most enzymes are proteins and perform the critical task of lowering the activation energies of chemical reactions inside the cell. Most of the reactions critical to a living cell happen too slowly at normal temperatures to be of any use to the cell. Without enzymes to speed up these reactions, life could not persist. Enzymes do this by binding to the reactant molecules and holding them in such a way as to make the chemical bond-breaking and -forming processes take place more easily. It is important to remember that enzymes do not change whether a reaction is exergonic (spontaneous) or endergonic. This is because they do not change the free energy of the reactants or products. They only reduce the activation energy required for the reaction to go forward (Figure **7.2.1.6**). In addition, an enzyme itself is unchanged by the reaction it catalyzes. Once one reaction has been catalyzed, the enzyme is able to participate in other reactions.

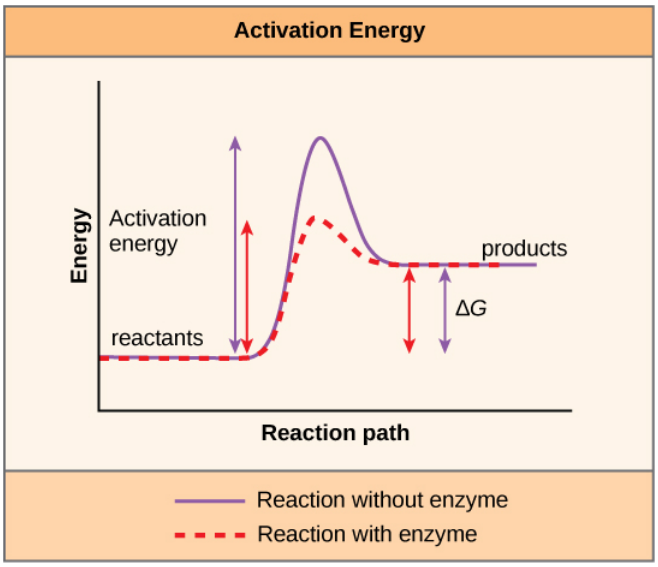


Figure 7.2.1.6: Enzymes lower the activation energy of the reaction but do not change the free energy of the reaction.

The chemical reactants to which an enzyme binds are called the enzyme's substrates. There may be one or more substrates, depending on the particular chemical reaction. In some reactions, a single reactant substrate is broken down into multiple products. In others, two substrates may come together to create one larger molecule. Two reactants might also enter a reaction and both become modified, but they leave the reaction as two products. The location within the enzyme where the substrate binds is called the enzyme's active site. The active site is where the "action" happens. Since enzymes are proteins, there is a unique combination of amino acid side chains within the active site. Each side chain is characterized by different properties. They can be large or small, weakly acidic or basic, hydrophilic or hydrophobic, positively or negatively charged, or neutral. The unique combination of side chains creates a very specific chemical environment within the active site. This specific environment is suited to bind to one specific chemical substrate (or substrates).

Active sites are subject to influences of the local environment. Increasing the environmental temperature generally increases reaction rates, enzyme-catalyzed or otherwise. However, temperatures outside of an optimal range reduce the rate at which an enzyme catalyzes a reaction. Hot temperatures will eventually cause enzymes to denature, an irreversible change in the three-dimensional shape and therefore the function of the enzyme. Enzymes are also suited to function best within a certain pH and salt concentration range, and, as with temperature, extreme pH, and salt concentrations can cause enzymes to denature.

For many years, scientists thought that enzyme-substrate binding took place in a simple "lock and key" fashion. This model asserted that the enzyme and substrate fit together perfectly in one instantaneous step. However, current research supports a model called induced fit (Figure **7.2.1.7**). The induced-fit model expands on the lock-and-key model by describing a more dynamic binding between enzyme and substrate. As the enzyme and substrate come together, their interaction causes a mild shift in the enzyme's structure that forms an ideal binding arrangement between enzyme and substrate.

CONCEPT IN ACTION



View an animation of induced fit.

When an enzyme binds its substrate, an enzyme-substrate complex is formed. This complex lowers the activation energy of the reaction and promotes its rapid progression in one of multiple possible ways. On a basic level, enzymes promote chemical reactions that involve more than one substrate by bringing the substrates together in an optimal orientation for reaction. Another way in which enzymes promote the reaction of their substrates is by creating an optimal environment within the active site for the reaction to occur. The chemical properties that emerge from the particular arrangement of amino acid R groups within an active site create the perfect environment for an enzyme's specific substrates to react.

The enzyme-substrate complex can also lower activation energy by compromising the bond structure so that it is easier to break. Finally, enzymes can also lower activation energies by taking part in the chemical reaction itself. In these cases, it is important to remember that the enzyme will always return to its original state by the completion of the reaction. One of the hallmark properties of enzymes is that they remain ultimately unchanged by the reactions they catalyze. After an enzyme has catalyzed a reaction, it releases its product(s) and can catalyze a new reaction.

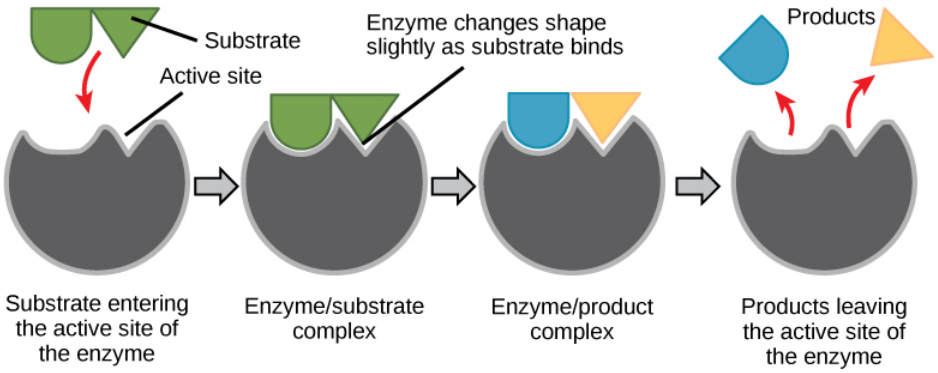


Figure 7.2.1.7: The induced-fit model is an adjustment to the lock-and-key model and explains how enzymes and substrates undergo dynamic modifications during the transition state to increase the affinity of the substrate for the active site.

It would seem ideal to have a scenario in which all of an organism's enzymes existed in abundant supply and functioned optimally under all cellular conditions, in all cells, at all times. However, a variety of mechanisms ensures that this does not happen. Cellular needs and conditions constantly vary from cell to cell, and change within individual cells over time. The required enzymes of stomach cells differ from those of fat storage cells, skin cells, blood cells, and nerve cells. Furthermore, a digestive organ cell works much harder to process and break down nutrients during the time that closely follows a meal compared with many hours after a meal. As these cellular demands and conditions vary, so must the amounts and functionality of different enzymes.

Since the rates of biochemical reactions are controlled by activation energy, and enzymes lower and determine activation energies for chemical reactions, the relative amounts and functioning of the variety of enzymes within a cell ultimately determine which reactions will proceed and at what rates. This determination is tightly controlled in cells. In certain cellular environments, enzyme activity is partly controlled by environmental factors like pH, temperature, salt concentration, and, in some cases, cofactors or coenzymes.

Enzymes can also be regulated in ways that either promote or reduce enzyme activity. There are many kinds of molecules that inhibit or promote enzyme function, and various mechanisms by which they do so. In some cases of enzyme inhibition, an inhibitor molecule is similar enough to a substrate that it can bind to the active site and simply block the substrate from binding. When this happens, the enzyme is inhibited through competitive inhibition, because an inhibitor molecule competes with the substrate for binding to the active site.

On the other hand, in noncompetitive inhibition, an inhibitor molecule binds to the enzyme in a location other than the active site, called an allosteric site, but still manages to block substrate binding to the active site. Some inhibitor molecules bind to enzymes in a location where their binding induces a conformational change that reduces the affinity of the enzyme for its substrate. This type of inhibition is called allosteric inhibition(Figure **7.2.1.8**). Most allosterically regulated enzymes are made up of more than one polypeptide, meaning that they have more than one protein subunit. When an allosteric inhibitor binds to a region on an enzyme, all active sites on the protein subunits are changed slightly such that they bind their substrates with less efficiency. There are allosteric activators as well as inhibitors. Allosteric activators bind to locations on an enzyme away from the active site, inducing a conformational change that increases the affinity of the enzyme's active site(s) for its substrate(s) (Figure **7.2.1.8**).

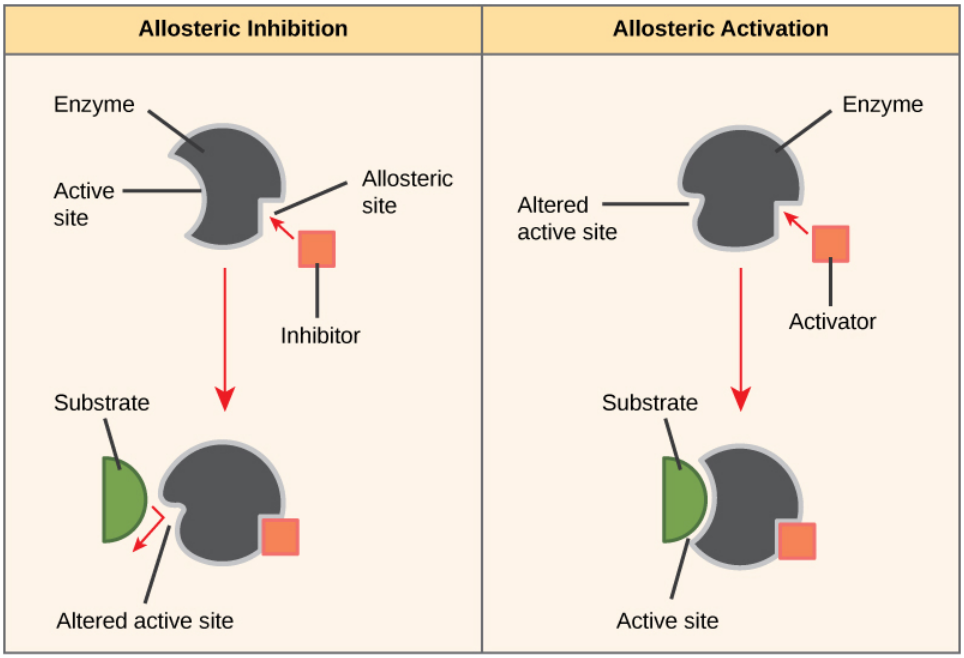


Figure 7.2.1.8: Allosteric inhibition works by indirectly inducing a conformational change to the active site such that the substrate no longer fits. In contrast, in allosteric activation, the activator molecule modifies the shape of the active site to allow a better fit of the substrate.

CAREERS IN ACTION: Pharmaceutical Drug Developer



Figure 7.2.1.9: Have you ever wondered how pharmaceutical drugs are developed? (credit: Deborah Austin)

Enzymes are key components of metabolic pathways. Understanding how enzymes work and how they can be regulated are key principles behind the development of many of the pharmaceutical drugs on the market today. Biologists working in this field collaborate with other scientists to design drugs (Figure **7.2.1.9**).

Consider statins for example—statins is the name given to one class of drugs that can reduce cholesterol levels. These compounds are inhibitors of the enzyme HMG-CoA reductase, which is the enzyme that synthesizes cholesterol from lipids in the body. By inhibiting this enzyme, the level of cholesterol synthesized in the body can be reduced. Similarly, acetaminophen, popularly marketed under the brand name Tylenol, is an inhibitor of the enzyme cyclooxygenase. While it is used to provide relief from fever and inflammation (pain), its mechanism of action is still not completely understood.

How are drugs discovered? One of the biggest challenges in drug discovery is identifying a drug target. A drug target is a molecule that is literally the target of the drug. In the case of statins, HMG-CoA reductase is the drug target. Drug targets are identified through painstaking research in the laboratory. Identifying the target alone is not enough; scientists also need to know how the target acts inside the cell and which reactions go awry in the case of disease. Once the target and the pathway are identified, then the actual process of drug design begins. In this stage, chemists and biologists work together to design and synthesize molecules that can block or activate a particular reaction. However, this is only the beginning: If and when a drug

prototype is successful in performing its function, then it is subjected to many tests from in vitro experiments to clinical trials before it can get approval from the U.S. Food and Drug Administration to be on the market.

Many enzymes do not work optimally, or even at all, unless bound to other specific non-protein helper molecules. They may bond either temporarily through ionic or hydrogen bonds, or permanently through stronger covalent bonds. Binding to these molecules promotes optimal shape and function of their respective enzymes. Two examples of these types of helper molecules are cofactors and coenzymes. Cofactors are inorganic ions such as ions of iron and magnesium. Coenzymes are organic helper molecules, those with a basic atomic structure made up of carbon and hydrogen. Like enzymes, these molecules participate in reactions without being changed themselves and are ultimately recycled and reused. Vitamins are the source of coenzymes. Some vitamins are the precursors of coenzymes and others act directly as coenzymes. Vitamin C is a direct coenzyme for multiple enzymes that take part in building the important connective tissue, collagen. Therefore, enzyme function is, in part, regulated by the abundance of various cofactors and coenzymes, which may be supplied by an organism's diet or, in some cases, produced by the organism.

Feedback Inhibition in Metabolic Pathways

Molecules can regulate enzyme function in many ways. The major question remains, however: What are these molecules and where do they come from? Some are cofactors and coenzymes, as you have learned. What other molecules in the cell provide enzymatic regulation such as allosteric modulation, and competitive and non-competitive inhibition? Perhaps the most relevant sources of regulatory molecules, with respect to enzymatic cellular metabolism, are the products of the cellular metabolic reactions themselves. In a most efficient and elegant way, cells have evolved to use the products of their own reactions for feedback inhibition of enzyme activity. Feedback inhibition involves the use of a reaction product to regulate its own further production (Figure **7.2.1.10**). The cell responds to an abundance of the products by slowing down production during anabolic or catabolic reactions. Such reaction products may inhibit the enzymes that catalyzed their production through the mechanisms described above.

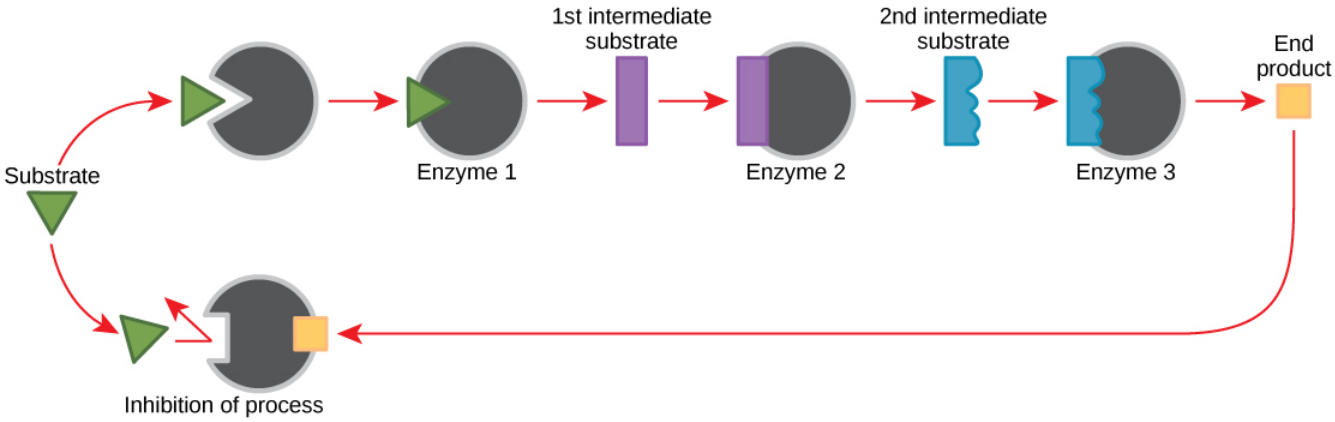


Figure 7.2.1.10: Metabolic pathways are a series of reactions catalyzed by multiple enzymes. Feedback inhibition, where the end product of the pathway inhibits an upstream process, is an important regulatory mechanism in cells.

The production of both amino acids and nucleotides is controlled through feedback inhibition. Additionally, ATP is an allosteric regulator of some of the enzymes involved in the catabolic breakdown of sugar, the process that creates ATP. In this way, when ATP is in abundant supply, the cell can prevent the production of ATP. On the other hand, ADP serves as a positive allosteric regulator (an allosteric activator) for some of the same enzymes that are inhibited by ATP. Thus, when relative levels of ADP are high compared to ATP, the cell is triggered to produce more ATP through sugar catabolism.

Section Summary

Cells perform the functions of life through various chemical reactions. A cell's metabolism refers to the combination of chemical reactions that take place within it. Catabolic reactions break down complex chemicals into simpler ones and are associated with energy release. Anabolic processes build complex molecules out of simpler ones and require energy.

In studying energy, the term system refers to the matter and environment involved in energy transfers. Entropy is a measure of the disorder of a system. The physical laws that describe the transfer of energy are the laws of thermodynamics. The first law states that the total amount of energy in the universe is constant. The second law of thermodynamics states that every energy transfer involves

some loss of energy in an unusable form, such as heat energy. Energy comes in different forms: kinetic, potential, and free. The change in free energy of a reaction can be negative (releases energy, exergonic) or positive (consumes energy, endergonic). All reactions require an initial input of energy to proceed, called the activation energy.

Enzymes are chemical catalysts that speed up chemical reactions by lowering their activation energy. Enzymes have an active site with a unique chemical environment that fits particular chemical reactants for that enzyme, called substrates. Enzymes and substrates are thought to bind according to an induced-fit model. Enzyme action is regulated to conserve resources and respond optimally to the environment.

Art Connections

Figure 7.2.1.5: Look at each of the processes shown and decide if it is endergonic or exergonic.

Answer

A compost pile decomposing is an exergonic process. A baby developing from a fertilized egg is an endergonic process. Tea dissolving into water is an exergonic process. A ball rolling downhill is an exergonic process.

Glossary

activation energy
the amount of initial energy necessary for reactions to occur

active site
a specific region on the enzyme where the substrate binds

allosteric inhibition
the mechanism for inhibiting enzyme action in which a regulatory molecule binds to a second site (not the active site) and initiates a conformation change in the active site, preventing binding with the substrate

anabolic
describes the pathway that requires a net energy input to synthesize complex molecules from simpler ones

bioenergetics
the concept of energy flow through living systems

catabolic
describes the pathway in which complex molecules are broken down into simpler ones, yielding energy as an additional product of the reaction

competitive inhibition
a general mechanism of enzyme activity regulation in which a molecule other than the enzyme's substrate is able to bind the active site and prevent the substrate itself from binding, thus inhibiting the overall rate of reaction for the enzyme

endergonic
describes a chemical reaction that results in products that store more chemical potential energy than the reactants

enzyme
a molecule that catalyzes a biochemical reaction

exergonic
describes a chemical reaction that results in products with less chemical potential energy than the reactants, plus the release of free energy

feedback inhibition

a mechanism of enzyme activity regulation in which the product of a reaction or the final product of a series of sequential reactions inhibits an enzyme for an earlier step in the reaction series

heat energy
the energy transferred from one system to another that is not work

kinetic energy
the type of energy associated with objects in motion

metabolism
all the chemical reactions that take place inside cells, including those that use energy and those that release energy

noncompetitive inhibition
a general mechanism of enzyme activity regulation in which a regulatory molecule binds to a site other than the active site and prevents the active site from binding the substrate; thus, the inhibitor molecule does not compete with the substrate for the active site; allosteric inhibition is a form of noncompetitive inhibition

potential energy
the type of energy that refers to the potential to do work

substrate
a molecule on which the enzyme acts

thermodynamics
the science of the relationships between heat, energy, and work

Contributors and Attributions

- Samantha Fowler (Clayton State University), Rebecca Roush (Sandhills Community College), James Wise (Hampton University). Original content by OpenStax (CC BY 4.0; Access for free at https://cnx.org/contents/b3c1e1d2-83...4-e119a8aafbdd).

This page titled 7.2.1: Energy and Metabolism is shared under a CC BY license and was authored, remixed, and/or curated by .

- **4.1: Energy and Metabolism** by OpenStax is licensed CC BY 4.0.

7.2.2: Glycolysis

Even exergonic, energy-releasing reactions require a small amount of activation energy to proceed. However, consider endergonic reactions, which require much more energy input because their products have more free energy than their reactants. Within the cell, where does energy to power such reactions come from? The answer lies with an energy-supplying molecule called adenosine triphosphate, or ATP. ATP is a small, relatively simple molecule, but within its bonds contains the potential for a quick burst of energy that can be harnessed to perform cellular work. This molecule can be thought of as the primary energy currency of cells in the same way that money is the currency that people exchange for things they need. ATP is used to power the majority of energy-requiring cellular reactions.

ATP in Living Systems

A living cell cannot store significant amounts of free energy. Excess free energy would result in an increase of heat in the cell, which would denature enzymes and other proteins, and thus destroy the cell. Rather, a cell must be able to store energy safely and release it for use only as needed. Living cells accomplish this using ATP, which can be used to fill any energy need of the cell. How? It functions as a rechargeable battery.

When ATP is broken down, usually by the removal of its terminal phosphate group, energy is released. This energy is used to do work by the cell, usually by the binding of the released phosphate to another molecule, thus activating it. For example, in the mechanical work of muscle contraction, ATP supplies energy to move the contractile muscle proteins.

ATP Structure and Function

At the heart of ATP is a molecule of adenosine monophosphate (AMP), which is composed of an adenine molecule bonded to both a ribose molecule and a single phosphate group (Figure **7.2.2.1**). Ribose is a five-carbon sugar found in RNA and AMP is one of the nucleotides in RNA. The addition of a second phosphate group to this core molecule results in adenosine diphosphate (ADP); the addition of a third phosphate group forms adenosine triphosphate (ATP).

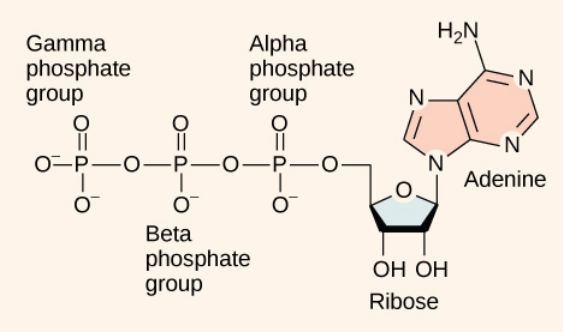


Figure 7.2.2.1: The structure of ATP shows the basic components of a two-ring adenine, five-carbon ribose, and three phosphate groups.

The addition of a phosphate group to a molecule requires a high amount of energy and results in a high-energy bond. Phosphate groups are negatively charged and thus repel one another when they are arranged in series, as they are in ADP and ATP. This repulsion makes the ADP and ATP molecules inherently unstable. The release of one or two phosphate groups from ATP, a process called hydrolysis, releases energy.

Glycolysis

You have read that nearly all of the energy used by living things comes to them in the bonds of the sugar, glucose. Glycolysis is the first step in the breakdown of glucose to extract energy for cell metabolism. Many living organisms carry out glycolysis as part of their metabolism. Glycolysis takes place in the cytoplasm of most prokaryotic and all eukaryotic cells.

Glycolysis begins with the six-carbon, ring-shaped structure of a single glucose molecule and ends with two molecules of a three-carbon sugar called pyruvate. Glycolysis consists of two distinct phases. In the first part of the glycolysis pathway, energy is used to make adjustments so that the six-carbon sugar molecule can be split evenly into two three-carbon pyruvate molecules. In the second part of glycolysis, ATP and nicotinamide-adenine dinucleotide (NADH) are produced (Figure **7.2.2.2**).

If the cell cannot catabolize the pyruvate molecules further, it will harvest only two ATP molecules from one molecule of glucose. For example, mature mammalian red blood cells are only capable of glycolysis, which is their sole source of ATP. If glycolysis is interrupted, these cells would eventually die.

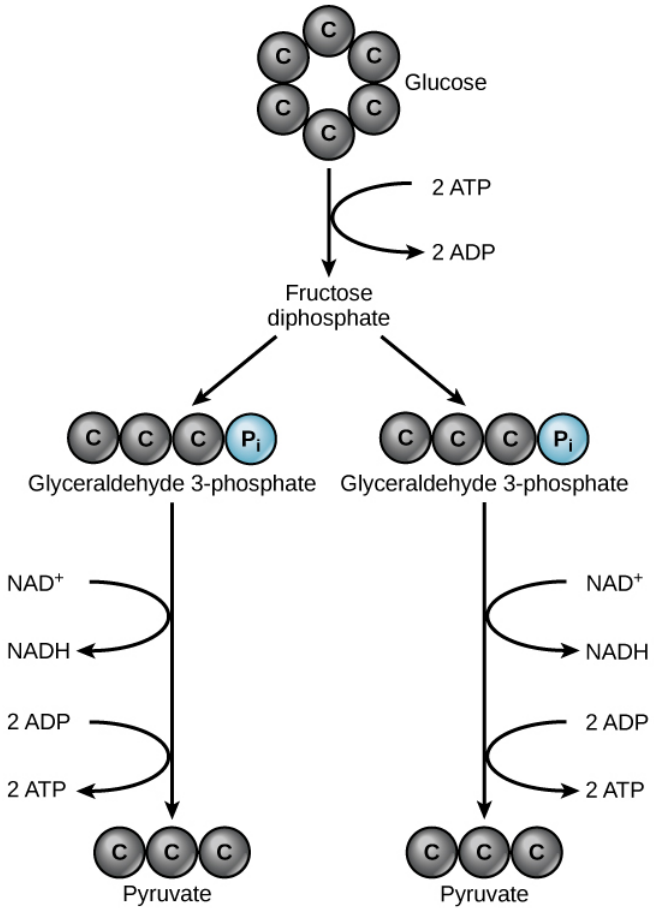


Figure 7.2.2.2: In glycolysis, a glucose molecule is converted into two pyruvate molecules.

Summary

ATP functions as the energy currency for cells. It allows cells to store energy briefly and transport it within itself to support endergonic chemical reactions. The structure of ATP is that of an RNA nucleotide with three phosphate groups attached. As ATP is used for energy, a phosphate group is detached, and ADP is produced. Energy derived from glucose catabolism is used to recharge ADP into ATP.

Glycolysis is the first pathway used in the breakdown of glucose to extract energy. Because it is used by nearly all organisms on earth, it must have evolved early in the history of life. Glycolysis consists of two parts: The first part prepares the six-carbon ring of glucose for separation into two three-carbon sugars. Energy from ATP is invested into the molecule during this step to energize the separation. The second half of glycolysis extracts ATP and high-energy electrons from hydrogen atoms and attaches them to NAD^+. Two ATP molecules are invested in the first half and four ATP molecules are formed during the second half. This produces a net gain of two ATP molecules per molecule of glucose for the cell.

Glossary

ATP

(also, adenosine triphosphate) the cell's energy currency

glycolysis

the process of breaking glucose into two three-carbon molecules with the production of ATP and NADH

Contributors and Attributions

- Samantha Fowler (Clayton State University), Rebecca Roush (Sandhills Community College), James Wise (Hampton University). Original content by OpenStax (CC BY 4.0; Access for free at https://cnx.org/contents/b3c1e1d2-83...4-e119a8aafbdd).

This page titled 7.2.2: Glycolysis is shared under a CC BY license and was authored, remixed, and/or curated by .

- **4.2: Glycolysis** by OpenStax is licensed CC BY 4.0.

7.2.3: Citric Acid Cycle and Oxidative Phosphorylation

The Citric Acid Cycle

In eukaryotic cells, the pyruvate molecules produced at the end of glycolysis are transported into mitochondria, which are sites of cellular respiration. If oxygen is available, aerobic respiration will go forward. In mitochondria, pyruvate will be transformed into a two-carbon acetyl group (by removing a molecule of carbon dioxide) that will be picked up by a carrier compound called coenzyme A (CoA), which is made from vitamin B_5. The resulting compound is called acetyl CoA. (Figure **7.2.3.1**). Acetyl CoA can be used in a variety of ways by the cell, but its major function is to deliver the acetyl group derived from pyruvate to the next pathway in glucose catabolism.

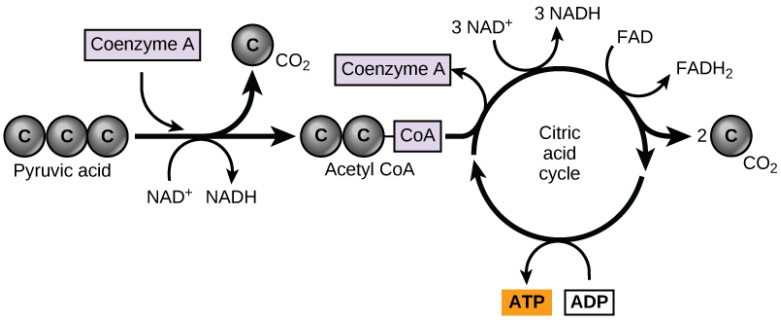


Figure 7.2.3.1: Pyruvate is converted into acetyl-CoA before entering the citric acid cycle.

Like the conversion of pyruvate to acetyl CoA, the citric acid cycle in eukaryotic cells takes place in the matrix of the mitochondria. Unlike glycolysis, the citric acid cycle is a closed loop: The last part of the pathway regenerates the compound used in the first step. The eight steps of the cycle are a series of chemical reactions that produces two carbon dioxide molecules, one ATP molecule (or an equivalent), and reduced forms (NADH and $FADH_2$) of NAD^+ and FAD^+, important coenzymes in the cell. Part of this is considered an aerobic pathway (oxygen-requiring) because the NADH and $FADH_2$ produced must transfer their electrons to the next pathway in the system, which will use oxygen. If oxygen is not present, this transfer does not occur.

Two carbon atoms come into the citric acid cycle from each acetyl group. Two carbon dioxide molecules are released on each turn of the cycle; however, these do not contain the same carbon atoms contributed by the acetyl group on that turn of the pathway. The two acetyl-carbon atoms will eventually be released on later turns of the cycle; in this way, all six carbon atoms from the original glucose molecule will be eventually released as carbon dioxide. It takes two turns of the cycle to process the equivalent of one glucose molecule. Each turn of the cycle forms three high-energy NADH molecules and one high-energy $FADH_2$ molecule. These high-energy carriers will connect with the last portion of aerobic respiration to produce ATP molecules. One ATP (or an equivalent) is also made in each cycle. Several of the intermediate compounds in the citric acid cycle can be used in synthesizing non-essential amino acids; therefore, the cycle is both anabolic and catabolic.

Oxidative Phosphorylation

You have just read about two pathways in glucose catabolism—glycolysis and the citric acid cycle—that generate ATP. Most of the ATP generated during the aerobic catabolism of glucose, however, is not generated directly from these pathways. Rather, it derives from a process that begins with passing electrons through a series of chemical reactions to a final electron acceptor, oxygen. These reactions take place in specialized protein complexes located in the inner membrane of the mitochondria of eukaryotic organisms and on the inner part of the cell membrane of prokaryotic organisms. The energy of the electrons is harvested and used to generate a electrochemical gradient across the inner mitochondrial membrane. The potential energy of this gradient is used to generate ATP. The entirety of this process is called oxidative phosphorylation.

The electron transport chain (Figure **7.2.3.2a**) is the last component of aerobic respiration and is the only part of metabolism that uses atmospheric oxygen. Oxygen continuously diffuses into plants for this purpose. In animals, oxygen enters the body through the respiratory system. Electron transport is a series of chemical reactions that resembles a bucket brigade in that electrons are passed rapidly from one component to the next, to the endpoint of the chain where oxygen is the final electron acceptor and water is produced. There are four complexes composed of proteins, labeled I through IV in Figure **7.2.3.2c**, and the aggregation of these

four complexes, together with associated mobile, accessory electron carriers, is called the electron transport chain. The electron transport chain is present in multiple copies in the inner mitochondrial membrane of eukaryotes and in the plasma membrane of prokaryotes. In each transfer of an electron through the electron transport chain, the electron loses energy, but with some transfers, the energy is stored as potential energy by using it to pump hydrogen ions across the inner mitochondrial membrane into the intermembrane space, creating an electrochemical gradient.

ART CONNECTION

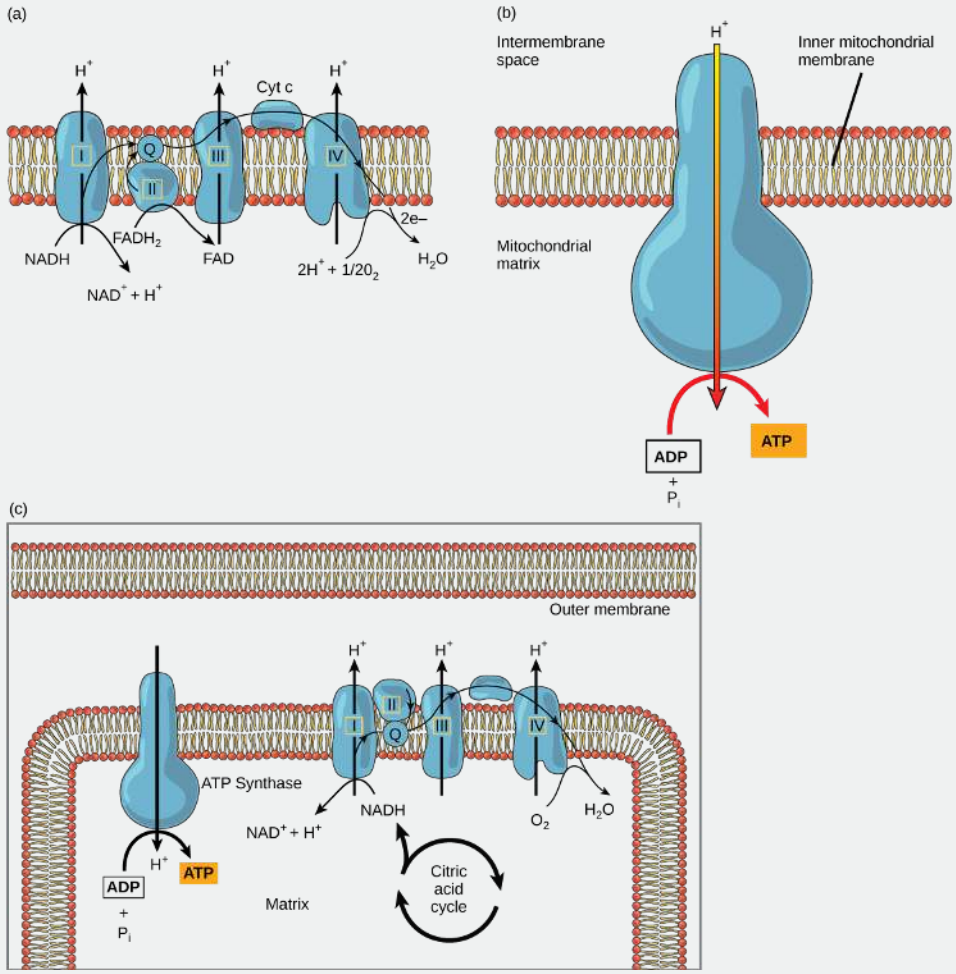


Figure 7.2.3.2: (a) The electron transport chain is a set of molecules that supports a series of oxidation-reduction reactions. (b) ATP synthase is a complex, molecular machine that uses an H^+ gradient to regenerate ATP from ADP. (c) Chemiosmosis relies on the potential energy provided by the H^+ gradient across the membrane.

Cyanide inhibits cytochrome c oxidase, a component of the electron transport chain. If cyanide poisoning occurs, would you expect the pH of the intermembrane space to increase or decrease? What affect would cyanide have on ATP synthesis?

Electrons from NADH and $FADH_2$ are passed to protein complexes in the electron transport chain. As they are passed from one complex to another (there are a total of four), the electrons lose energy, and some of that energy is used to pump hydrogen ions from the mitochondrial matrix into the intermembrane space. In the fourth protein complex, the electrons are accepted by oxygen, the terminal acceptor. The oxygen with its extra electrons then combines with two hydrogen ions, further enhancing the electrochemical gradient, to form water. If there were no oxygen present in the mitochondrion, the electrons could not be removed from the system, and the entire electron transport chain would back up and stop. The mitochondria would be unable to generate new ATP in this way, and the cell would ultimately die from lack of energy. This is the reason we must breathe to draw in new oxygen.

In the electron transport chain, the free energy from the series of reactions just described is used to pump hydrogen ions across the membrane. The uneven distribution of H^+ ions across the membrane establishes an electrochemical gradient, owing to the H^+ ions' positive charge and their higher concentration on one side of the membrane.

Hydrogen ions diffuse through the inner membrane through an integral membrane protein called ATP synthase (Figure **7.2.3.2b**). This complex protein acts as a tiny generator, turned by the force of the hydrogen ions diffusing through it, down their electrochemical gradient from the intermembrane space, where there are many mutually repelling hydrogen ions to the matrix, where there are few. The turning of the parts of this molecular machine regenerate ATP from ADP. This flow of hydrogen ions across the membrane through ATP synthase is called chemiosmosis.

Chemiosmosis (Figure **7.2.3.2c**) is used to generate 90 percent of the ATP made during aerobic glucose catabolism. The result of the reactions is the production of ATP from the energy of the electrons removed from hydrogen atoms. These atoms were originally part of a glucose molecule. At the end of the electron transport system, the electrons are used to reduce an oxygen molecule to oxygen ions. The extra electrons on the oxygen ions attract hydrogen ions (protons) from the surrounding medium, and water is formed. The electron transport chain and the production of ATP through chemiosmosis are collectively called oxidative phosphorylation.

ATP Yield

The number of ATP molecules generated from the catabolism of glucose varies. For example, the number of hydrogen ions that the electron transport chain complexes can pump through the membrane varies between species. Another source of variance stems from the shuttle of electrons across the mitochondrial membrane. The NADH generated from glycolysis cannot easily enter mitochondria. Thus, electrons are picked up on the inside of the mitochondria by either NAD^+ or FAD^+. Fewer ATP molecules are generated when FAD^+ acts as a carrier. NAD^+ is used as the electron transporter in the liver and FAD^+ in the brain, so ATP yield depends on the tissue being considered.

Another factor that affects the yield of ATP molecules generated from glucose is that intermediate compounds in these pathways are used for other purposes. Glucose catabolism connects with the pathways that build or break down all other biochemical compounds in cells, and the result is somewhat messier than the ideal situations described thus far. For example, sugars other than glucose are fed into the glycolytic pathway for energy extraction. Other molecules that would otherwise be used to harvest energy in glycolysis or the citric acid cycle may be removed to form nucleic acids, amino acids, lipids, or other compounds. Overall, in living systems, these pathways of glucose catabolism extract about 34 percent of the energy contained in glucose.

CAREERS IN ACTION: Mitochondrial Disease Physician

What happens when the critical reactions of cellular respiration do not proceed correctly? Mitochondrial diseases are genetic disorders of metabolism. Mitochondrial disorders can arise from mutations in nuclear or mitochondrial DNA, and they result in the production of less energy than is normal in body cells. Symptoms of mitochondrial diseases can include muscle weakness, lack of coordination, stroke-like episodes, and loss of vision and hearing. Most affected people are diagnosed in childhood, although there are some adult-onset diseases. Identifying and treating mitochondrial disorders is a specialized medical field. The educational preparation for this profession requires a college education, followed by medical school with a specialization in medical genetics. Medical geneticists can be board certified by the American Board of Medical Genetics and go on to become associated with professional organizations devoted to the study of mitochondrial disease, such as the Mitochondrial Medicine Society and the Society for Inherited Metabolic Disease.

Summary

The citric acid cycle is a series of chemical reactions that removes high-energy electrons and uses them in the electron transport chain to generate ATP. One molecule of ATP (or an equivalent) is produced per each turn of the cycle.

The electron transport chain is the portion of aerobic respiration that uses free oxygen as the final electron acceptor for electrons removed from the intermediate compounds in glucose catabolism. The electrons are passed through a series of chemical reactions, with a small amount of free energy used at three points to transport hydrogen ions across the membrane. This contributes to the gradient used in chemiosmosis. As the electrons are passed from NADH or $FADH_2$ down the electron transport chain, they lose energy. The products of the electron transport chain are water and ATP. A number of intermediate compounds can be diverted into

the anabolism of other biochemical molecules, such as nucleic acids, non-essential amino acids, sugars, and lipids. These same molecules, except nucleic acids, can serve as energy sources for the glucose pathway.

Art Connections

Figure 7.2.3.2: Cyanide inhibits cytochrome c oxidase, a component of the electron transport chain. If cyanide poisoning occurs, would you expect the pH of the intermembrane space to increase or decrease? What affect would cyanide have on ATP synthesis?

Answer

After cyanide poisoning, the electron transport chain can no longer pump electrons into the intermembrane space. The pH of the intermembrane space would increase, and ATP synthesis would stop.

Glossary

acetyl CoA
the combination of an acetyl group derived from pyruvic acid and coenzyme A which is made from pantothenic acid (a B-group vitamin)

ATP synthase
a membrane-embedded protein complex that regenerates ATP from ADP with energy from protons diffusing through it

chemiosmosis
the movement of hydrogen ions down their electrochemical gradient across a membrane through ATP synthase to generate ATP

citric acid cycle
a series of enzyme-catalyzed chemical reactions of central importance in all living cells that harvests the energy in carbon-carbon bonds of sugar molecules to generate ATP; the citric acid cycle is an aerobic metabolic pathway because it requires oxygen in later reactions to proceed

electron transport chain
a series of four large, multi-protein complexes embedded in the inner mitochondrial membrane that accepts electrons from donor compounds and harvests energy from a series of chemical reactions to generate a hydrogen ion gradient across the membrane

oxidative phosphorylation
the production of ATP by the transfer of electrons down the electron transport chain to create a proton gradient that is used by ATP synthase to add phosphate groups to ADP molecules

Contributors and Attributions

- Samantha Fowler (Clayton State University), Rebecca Roush (Sandhills Community College), James Wise (Hampton University). Original content by OpenStax (CC BY 4.0; Access for free at https://cnx.org/contents/b3c1e1d2-83...4-e119a8aafbdd).

This page titled 7.2.3: Citric Acid Cycle and Oxidative Phosphorylation is shared under a CC BY license and was authored, remixed, and/or curated by .

- **4.3: Citric Acid Cycle and Oxidative Phosphorylation** by OpenStax is licensed CC BY 4.0.

7.2.4: Fermentation

In aerobic respiration, the final electron acceptor is an oxygen molecule, O_2. If aerobic respiration occurs, then ATP will be produced using the energy of the high-energy electrons carried by NADH or $FADH_2$ to the electron transport chain. If aerobic respiration does not occur, NADH must be reoxidized to NAD^+ for reuse as an electron carrier for glycolysis to continue. How is this done? Some living systems use an organic molecule as the final electron acceptor. Processes that use an organic molecule to regenerate NAD^+ from NADH are collectively referred to as fermentation. In contrast, some living systems use an inorganic molecule (other than oxygen) as a final electron acceptor to regenerate NAD^+; both methods are anaerobic (do not require oxygen) to achieve NAD^+ regeneration and enable organisms to convert energy for their use in the absence of oxygen.

Lactic Acid Fermentation

The fermentation method used by animals and some bacteria like those in yogurt is lactic acid fermentation (Figure **7.2.4.1**). This occurs routinely in mammalian red blood cells and in skeletal muscle that has insufficient oxygen supply to allow aerobic respiration to continue (that is, in muscles used to the point of fatigue). In muscles, lactic acid produced by fermentation must be removed by the blood circulation and brought to the liver for further metabolism. The chemical reaction of lactic acid fermentation is the following:

$$\text{Pyruvic acid} + \text{NADH} \leftrightarrow \text{lactic acid} + \text{NAD}^+$$

The enzyme that catalyzes this reaction is lactate dehydrogenase. The reaction can proceed in either direction, but the left-to-right reaction is inhibited by acidic conditions. This lactic acid build-up causes muscle stiffness and fatigue. Once the lactic acid has been removed from the muscle and is circulated to the liver, it can be converted back to pyruvic acid and further catabolized for energy.

ART CONNECTION

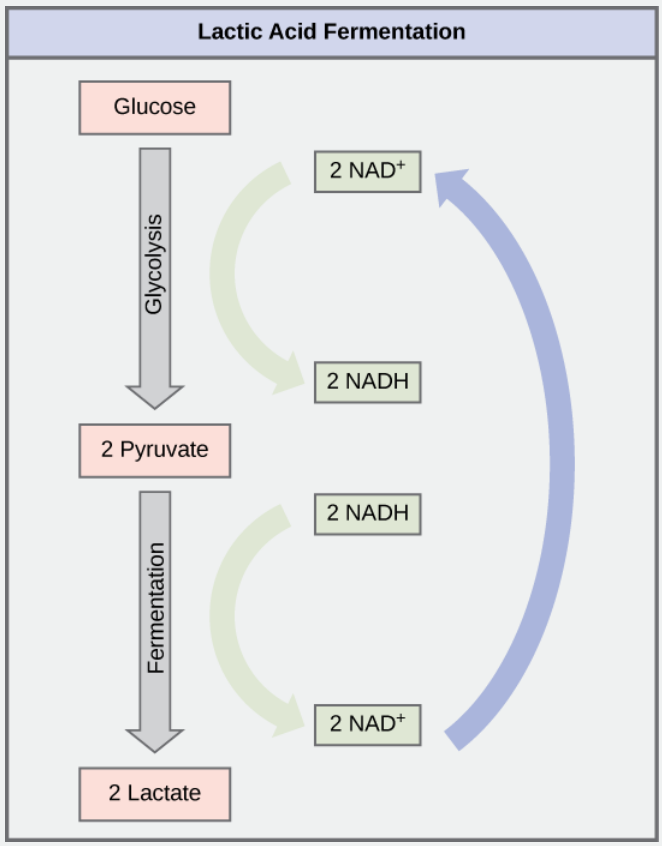


Figure 7.2.4.1: Lactic acid fermentation is common in muscles that have become exhausted by use.

> Tremetol, a metabolic poison found in white snake root plant, prevents the metabolism of lactate. When cows eat this plant, Tremetol is concentrated in the milk. Humans who consume the milk become ill. Symptoms of this disease, which include vomiting, abdominal pain, and tremors, become worse after exercise. Why do you think this is the case?

Alcohol Fermentation

Another familiar fermentation process is alcohol fermentation (Figure **7.2.4.2**), which produces ethanol, an alcohol. The alcohol fermentation reaction is the following:

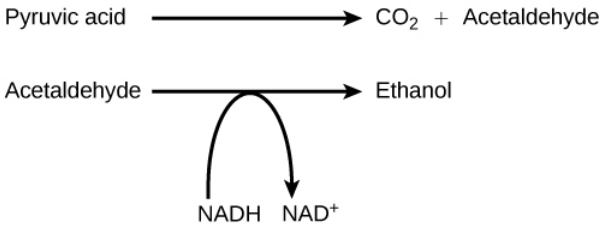


Figure 7.2.4.2: The reaction resulting in alcohol fermentation is shown.

In the first reaction, a carboxyl group is removed from pyruvic acid, releasing carbon dioxide as a gas. The loss of carbon dioxide reduces the molecule by one carbon atom, making acetaldehyde. The second reaction removes an electron from NADH, forming NAD^+ and producing ethanol from the acetaldehyde, which accepts the electron. The fermentation of pyruvic acid by yeast produces the ethanol found in alcoholic beverages (Figure **7.2.4.3**). If the carbon dioxide produced by the reaction is not vented from the fermentation chamber, for example in beer and sparkling wines, it remains dissolved in the medium until the pressure is released. Ethanol above 12 percent is toxic to yeast, so natural levels of alcohol in wine occur at a maximum of 12 percent.



Figure 7.2.4.3: Fermentation of grape juice to make wine produces CO_2 as a byproduct. Fermentation tanks have valves so that pressure inside the tanks can be released.

Anaerobic Cellular Respiration

Certain prokaryotes, including some species of bacteria and Archaea, use anaerobic respiration. For example, the group of Archaea called methanogens reduces carbon dioxide to methane to oxidize NADH. These microorganisms are found in soil and in the digestive tracts of ruminants, such as cows and sheep. Similarly, sulfate-reducing bacteria and Archaea, most of which are anaerobic (Figure **7.2.4.4**), reduce sulfate to hydrogen sulfide to regenerate NAD^+ from NADH.

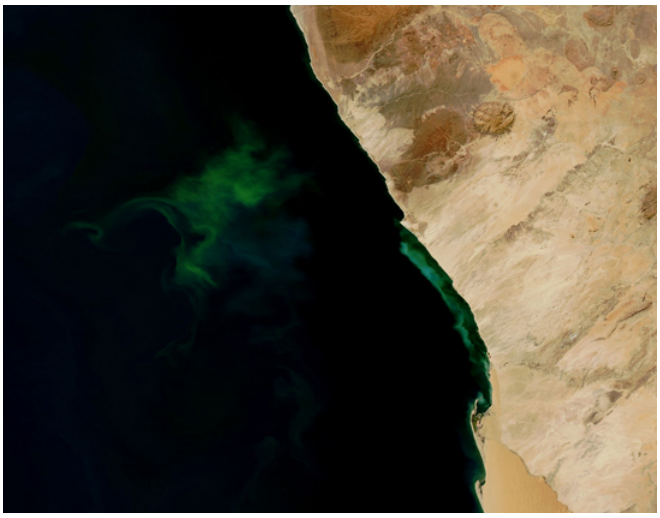


Figure 7.2.4.4: The green color seen in these coastal waters is from an eruption of hydrogen sulfide. Anaerobic, sulfate-reducing bacteria release hydrogen sulfide gas as they decompose algae in the water. (credit: NASA image courtesy Jeff Schmaltz, MODIS Land Rapid Response Team at NASA GSFC)

CONCEPT IN ACTION



Visit this site to see anaerobic cellular respiration in action.

Other fermentation methods occur in bacteria. Many prokaryotes are facultatively anaerobic. This means that they can switch between aerobic respiration and fermentation, depending on the availability of oxygen. Certain prokaryotes, like *Clostridia* bacteria, are obligate anaerobes. Obligate anaerobes live and grow in the absence of molecular oxygen. Oxygen is a poison to these microorganisms and kills them upon exposure. It should be noted that all forms of fermentation, except lactic acid fermentation, produce gas. The production of particular types of gas is used as an indicator of the fermentation of specific carbohydrates, which plays a role in the laboratory identification of the bacteria. The various methods of fermentation are used by different organisms to ensure an adequate supply of NAD^+ for the sixth step in glycolysis. Without these pathways, that step would not occur, and no ATP would be harvested from the breakdown of glucose.

Section Summary

If NADH cannot be metabolized through aerobic respiration, another electron acceptor is used. Most organisms will use some form of fermentation to accomplish the regeneration of NAD^+, ensuring the continuation of glycolysis. The regeneration of NAD^+ in fermentation is not accompanied by ATP production; therefore, the potential for NADH to produce ATP using an electron transport chain is not utilized.

Art Connections

Figure **7.2.4.1**: Tremetol, a metabolic poison found in white snake root plant, prevents the metabolism of lactate. When cows eat this plant, Tremetol is concentrated in the milk. Humans who consume the milk become ill. Symptoms of this disease, which include vomiting, abdominal pain, and tremors, become worse after exercise. Why do you think this is the case?

Answer

The illness is caused by lactic acid build-up. Lactic acid levels rise after exercise, making the symptoms worse. Milk sickness is rare today, but was common in the Midwestern United States in the early 1800s.

Glossary

anaerobic cellular respiration

the use of an electron acceptor other than oxygen to complete metabolism using electron transport-based chemiosmosis

fermentation

the steps that follow the partial oxidation of glucose via glycolysis to regenerate NAD^+; occurs in the absence of oxygen and uses an organic compound as the final electron acceptor

Contributors and Attributions

- Samantha Fowler (Clayton State University), Rebecca Roush (Sandhills Community College), James Wise (Hampton University). Original content by OpenStax (CC BY 4.0; Access for free at https://cnx.org/contents/b3c1e1d2-83...4-e119a8aafbdd).

This page titled 7.2.4: Fermentation is shared under a CC BY license and was authored, remixed, and/or curated by .

- **4.4: Fermentation** by OpenStax is licensed CC BY 4.0.

7.2.5: Connections to Other Metabolic Pathways

You have learned about the catabolism of glucose, which provides energy to living cells. But living things consume more than just glucose for food. How does a turkey sandwich, which contains protein, provide energy to your cells? This happens because all of the catabolic pathways for carbohydrates, proteins, and lipids eventually connect into glycolysis and the citric acid cycle pathways (Figure **7.2.5.1**). Metabolic pathways should be thought of as porous—that is, substances enter from other pathways, and other substances leave for other pathways. These pathways are not closed systems. Many of the products in a particular pathway are reactants in other pathways.

Connections of Other Sugars to Glucose Metabolism

Glycogen, a polymer of glucose, is a short-term energy storage molecule in animals. When there is adequate ATP present, excess glucose is converted into glycogen for storage. Glycogen is made and stored in the liver and muscle. Glycogen will be taken out of storage if blood sugar levels drop. The presence of glycogen in muscle cells as a source of glucose allows ATP to be produced for a longer time during exercise.

Sucrose is a disaccharide made from glucose and fructose bonded together. Sucrose is broken down in the small intestine, and the glucose and fructose are absorbed separately. Fructose is one of the three dietary monosaccharides, along with glucose and galactose (which is part of milk sugar, the disaccharide lactose), that are absorbed directly into the bloodstream during digestion. The catabolism of both fructose and galactose produces the same number of ATP molecules as glucose.

Connections of Proteins to Glucose Metabolism

Proteins are broken down by a variety of enzymes in cells. Most of the time, amino acids are recycled into new proteins. If there are excess amino acids, however, or if the body is in a state of famine, some amino acids will be shunted into pathways of glucose catabolism. Each amino acid must have its amino group removed prior to entry into these pathways. The amino group is converted into ammonia. In mammals, the liver synthesizes urea from two ammonia molecules and a carbon dioxide molecule. Thus, urea is the principal waste product in mammals from the nitrogen originating in amino acids, and it leaves the body in urine.

Connections of Lipids to Glucose Metabolism

The lipids that are connected to the glucose pathways are cholesterol and triglycerides. Cholesterol is a lipid that contributes to cell membrane flexibility and is a precursor of steroid hormones. The synthesis of cholesterol starts with acetyl CoA and proceeds in only one direction. The process cannot be reversed, and ATP is not produced.

Triglycerides are a form of long-term energy storage in animals. Triglycerides store about twice as much energy as carbohydrates. Triglycerides are made of glycerol and three fatty acids. Animals can make most of the fatty acids they need. Triglycerides can be both made and broken down through parts of the glucose catabolism pathways. Glycerol can be phosphorylated and proceeds through glycolysis. Fatty acids are broken into two-carbon units that enter the citric acid cycle.

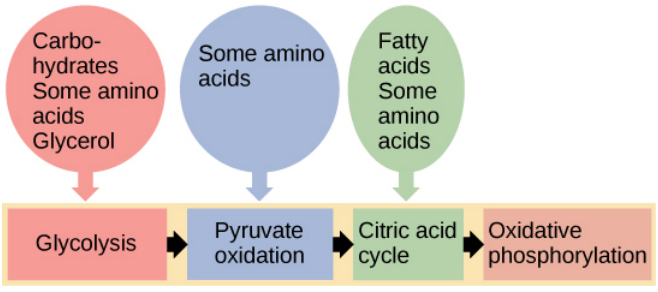


Figure 7.2.5.1: Glycogen from the liver and muscles, together with fats, can feed into the catabolic pathways for carbohydrates.

EVOLUTION IN ACTION: Pathways of Photosynthesis and Cellular Metabolism

Photosynthesis and cellular metabolism consist of several very complex pathways. It is generally thought that the first cells arose in an aqueous environment—a “soup” of nutrients. If these cells reproduced successfully and their numbers climbed steadily, it follows that the cells would begin to deplete the nutrients from the medium in which they lived, as they shifted the

nutrients into their own cells. This hypothetical situation would have resulted in natural selection favoring those organisms that could exist by using the nutrients that remained in their environment and by manipulating these nutrients into materials that they could use to survive. Additionally, selection would favor those organisms that could extract maximal value from the available nutrients.

An early form of photosynthesis developed that harnessed the sun's energy using compounds other than water as a source of hydrogen atoms, but this pathway did not produce free oxygen. It is thought that glycolysis developed prior to this time and could take advantage of simple sugars being produced, but these reactions were not able to fully extract the energy stored in the carbohydrates. A later form of photosynthesis used water as a source of hydrogen ions and generated free oxygen. Over time, the atmosphere became oxygenated. Living things adapted to exploit this new atmosphere and allowed respiration as we know it to evolve. When the full process of photosynthesis as we know it developed and the atmosphere became oxygenated, cells were finally able to use the oxygen expelled by photosynthesis to extract more energy from the sugar molecules using the citric acid cycle.

Summary

The breakdown and synthesis of carbohydrates, proteins, and lipids connect with the pathways of glucose catabolism. The carbohydrates that can also feed into glucose catabolism include galactose, fructose, and glycogen. These connect with glycolysis. The amino acids from proteins connect with glucose catabolism through pyruvate, acetyl CoA, and components of the citric acid cycle. Cholesterol synthesis starts with acetyl CoA, and the components of triglycerides are picked up by acetyl CoA and enter the citric acid cycle.

Contributors and Attributions

- Samantha Fowler (Clayton State University), Rebecca Roush (Sandhills Community College), James Wise (Hampton University). Original content by OpenStax (CC BY 4.0; Access for free at https://cnx.org/contents/b3c1e1d2-83...4-e119a8aafbdd).

This page titled 7.2.5: Connections to Other Metabolic Pathways is shared under a CC BY license and was authored, remixed, and/or curated by .

- **4.5: Connections to Other Metabolic Pathways** by OpenStax is licensed CC BY 4.0.

7.2.E: How Cells Obtain Energy (Exercises)

4.1: Energy and Metabolism

Cells perform the functions of life through various chemical reactions. A cell's metabolism refers to the combination of chemical reactions that take place within it. Catabolic reactions break down complex chemicals into simpler ones and are associated with energy release. Anabolic processes build complex molecules out of simpler ones and require energy. In studying energy, the term system refers to the matter and environment involved in energy transfers.

Review Questions

Which of the following is not an example of an energy transformation?

A. Heating up dinner in a microwave
B. Solar panels at work
C. Formation of static electricity
D. None of the above

Answer

D

Which of the following is not true about enzymes?

A. They are consumed by the reactions they catalyze.
B. They are usually made of amino acids.
C. They lower the activation energy of chemical reactions.
D. Each one is specific to the particular substrate(s) to which it binds.

Answer

A

Free Response

Does physical exercise to increase muscle mass involve anabolic and/or catabolic processes? Give evidence for your answer.

Answer

Physical exercise involves both anabolic and catabolic processes. Body cells break down sugars to provide ATP to do the work necessary for exercise, such as muscle contractions. This is catabolism. Muscle cells also must repair muscle tissue damaged by exercise by building new muscle. This is anabolism.

Explain in your own terms the difference between a spontaneous reaction and one that occurs instantaneously, and what causes this difference.

Answer

A spontaneous reaction is one that has a negative ΔG and thus releases energy. However, a spontaneous reaction need not occur quickly or suddenly like an instantaneous reaction. It may occur over long periods of time due to a large energy of activation, which prevents the reaction from occurring quickly.

With regard to enzymes, why are vitamins and minerals necessary for good health? Give examples.

Answer

Most vitamins and minerals act as cofactors and coenzymes for enzyme action. Many enzymes require the binding of certain cofactors or coenzymes to be able to catalyze their reactions. Since enzymes catalyze many important reactions, it is critical to obtain sufficient vitamins and minerals from diet and supplements. Vitamin C (ascorbic acid) is a coenzyme necessary for the action of enzymes that build collagen.

4.2: Glycolysis

ATP functions as the energy currency for cells. It allows cells to store energy briefly and transport it within itself to support endergonic chemical reactions. The structure of ATP is that of an RNA nucleotide with three phosphate groups attached. As ATP is used for energy, a phosphate group is detached, and ADP is produced. Energy derived from glucose catabolism is used to recharge ADP into ATP. Glycolysis is the first pathway used in the breakdown of glucose to extract energy.

Multiple Choice

Energy is stored long-term in the bonds of _____ and used short-term to perform work from a(n) _____ molecule.

A. ATP : glucose
B. an anabolic molecule : catabolic molecule
C. glucose : ATP
D. a catabolic molecule : anabolic molecule

Answer

C

The energy currency used by cells is _____.

A. ATP
B. ADP
C. AMP
D. adenosine

Answer

A

The glucose that enters the glycolysis pathway is split into two molecules of _____.

A. ATP
B. phosphate
C. NADH
D. pyruvate

Answer

D

Free Response

Both prokaryotic and eukaryotic organisms carry out some form of glycolysis. How does that fact support or not support the assertion that glycolysis is one of the oldest metabolic pathways?

Answer

If glycolysis evolved relatively late, it likely would not be as universal in organisms as it is. It probably evolved in very primitive organisms and persisted, with the addition of other pathways of carbohydrate metabolism that evolved later.

4.3: Citric Acid Cycle and Oxidative Phosphorylation

The citric acid cycle is a series of chemical reactions that removes high-energy electrons and uses them in the electron transport chain to generate ATP. One molecule of ATP (or an equivalent) is produced per each turn of the cycle. The electron transport chain is the portion of aerobic respiration that uses free oxygen as the final electron acceptor for electrons removed from the intermediate compounds in glucose catabolism.

Multiple Choice

What do the electrons added to NAD^+ do?

A. They become part of a fermentation pathway.
B. They go to another pathway for ATP production.
C. They energize the entry of the acetyl group into the citric acid cycle.
D. They are converted into NADP.

Answer

B

Chemiosmosis involves

A. the movement of electrons across the cell membrane
B. the movement of hydrogen atoms across a mitochondrial membrane
C. the movement of hydrogen ions across a mitochondrial membran
D. the movement of glucose through the cell membrane

Answer

C

Free Response

We inhale oxygen when we breathe and exhale carbon dioxide. What is the oxygen used for and where does the carbon dioxide come from?

Answer

The oxygen we inhale is the final electron acceptor in the electron transport chain and allows aerobic respiration to proceed, which is the most efficient pathway for harvesting energy in the form of ATP from food molecules. The carbon dioxide we breathe out is formed during the citric acid cycle when the bonds in carbon compounds are broken.

4.4: Fermentation

If NADH cannot be metabolized through aerobic respiration, another electron acceptor is used. Most organisms will use some form of fermentation to accomplish the regeneration of NAD+, ensuring the continuation of glycolysis. The regeneration of NAD+ in fermentation is not accompanied by ATP production; therefore, the potential for NADH to produce ATP using an electron transport chain is not utilized.

Review Questions

Which of the following fermentation methods can occur in animal skeletal muscles?

A. lactic acid fermentation
B. alcohol fermentation
C. mixed acid fermentation
D. propionic fermentation

Answer

A

Free Response

When muscle cells run out of oxygen, what happens to the potential for energy extraction from sugars and what pathways do the cell use?

Answer

Without oxygen, oxidative phosphorylation and the citric acid cycle stop, so ATP is no longer generated through this mechanism, which extracts the greatest amount of energy from a sugar molecule. In addition, NADH accumulates, preventing glycolysis from going forward because of an absence of NAD^+. Lactic acid fermentation uses the electrons in NADH to generate lactic acid from pyruvate, which allows glycolysis to continue and thus a smaller amount of ATP can be generated by the cell.

4.5: Connections to Other Metabolic Pathways

Metabolic pathways should be thought of as porous—that is, substances enter from other pathways, and other substances leave for other pathways. These pathways are not closed systems. Many of the products in a particular pathway are reactants in other pathways.

Multiple Choice

The cholesterol synthesized by cells uses which component of the glycolytic pathway as a starting point?

A. glucose
B. acetyl CoA
C. pyruvate
D. carbon dioxide

Answer

B

Beta oxidation is _____.

A. the breakdown of sugars
B. the assembly of sugars
C. the breakdown of fatty acids
D. the removal of amino groups from amino acids

Answer

C

Free Response

Would you describe metabolic pathways as inherently wasteful or inherently economical, and why?

Answer

They are very economical. The substrates, intermediates, and products move between pathways and do so in response to finely tuned feedback inhibition loops that keep metabolism overall on an even keel. Intermediates in one pathway may occur in another, and they can move from one pathway to another fluidly in response to the needs of the cell.

This page titled 7.2.E: How Cells Obtain Energy (Exercises) is shared under a CC BY license and was authored, remixed, and/or curated by .

- **4.E: How Cells Obtain Energy (Exercises)** by OpenStax is licensed CC BY 4.0.

7.3: Section - place holder

This page titled 7.3: Section - place holder is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

8: Biotechnology and Other Applications of Biochemistry

This page is a placeholder created because the page was deleted, but has sub-pages.

This page titled 8: Biotechnology and Other Applications of Biochemistry is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

8.1: Section 1-

This page titled 8.1: Section 1- is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

8.2: Section 2-

This page titled 8.2: Section 2- is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

8.3: Section 3-

This page titled 8.3: Section 3- is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

8.4: Section 4-

This page titled 8.4: Section 4- is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

8.5: Section 5-

This page titled 8.5: Section 5- is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

8.6: Section 6-

This page titled 8.6: Section 6- is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

SECTION OVERVIEW

8.7: The Immune System and Disease

8.7.1: Viruses

8.7.2: Innate Immunity

8.7.3: Adaptive Immunity

8.7.4: Disruptions in the Immune System

8.7.E: The Immune System and Desease (Exercises)

Thumbnail: The biohazard symbol was developed by the Dow Chemical Company in 1966 for their containment products. It is used in the labeling of biological materials that carry a significant health risk. (Public Domain; Silsor).

This page titled 8.7: The Immune System and Disease is shared under a CC BY license and was authored, remixed, and/or curated by .

8.7.1: Viruses

No one knows exactly when viruses emerged or from where they came, since viruses do not leave historical footprints such as fossils. Modern viruses are thought to be a mosaic of bits and pieces of nucleic acids picked up from various sources along their respective evolutionary paths. Viruses are acellular, parasitic entities that are not classified within any domain because they are not considered alive. They have no plasma membrane, internal organelles, or metabolic processes, and they do not divide. Instead, they infect a host cell and use the host's replication processes to produce progeny virus particles. Viruses infect all forms of organisms including bacteria, archaea, fungi, plants, and animals. Living things grow, metabolize, and reproduce. Viruses replicate, but to do so, they are entirely dependent on their host cells. They do not metabolize or grow, but are assembled in their mature form.

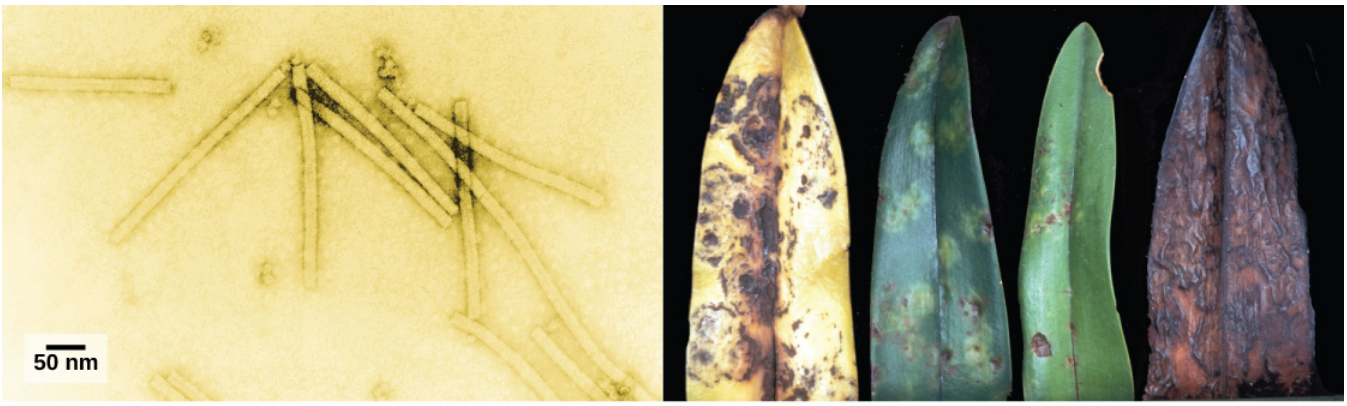


Figure 8.7.1.1: (a) The tobacco mosaic virus, seen by transmission electron microscopy, was the first virus to be discovered. (b) The leaves of an infected plant are shown. (credit a: scale-bar data from Matt Russell; credit b: modification of work by USDA, Department of Plant Pathology Archive, North Carolina State University)

Viruses are diverse. They vary in their structure, their replication methods, and in their target hosts or even host cells. While most biological diversity can be understood through evolutionary history, such as how species have adapted to conditions and environments, much about virus origins and evolution remains unknown.

How Viruses Replicate

Viruses were first discovered after the development of a porcelain filter, called the Chamberland-Pasteur filter, which could remove all bacteria visible under the microscope from any liquid sample. In 1886, Adolph Meyer demonstrated that a disease of tobacco plants, tobacco mosaic disease, could be transferred from a diseased plant to a healthy one through liquid plant extracts. In 1892, Dmitri Ivanowski showed that this disease could be transmitted in this way even after the Chamberland-Pasteur filter had removed all viable bacteria from the extract. Still, it was many years before it was proven that these "filterable" infectious agents were not simply very small bacteria but were a new type of tiny, disease-causing particle.

Virions, single virus particles, are very small, about 20–250 nanometers (1 nanometer = 1/1,000,000 mm). These individual virus particles are the infectious form of a virus outside the host cell. Unlike bacteria (which are about 100 times larger), we cannot see viruses with a light microscope, with the exception of some large virions of the poxvirus family (Figure 8.7.1.2).

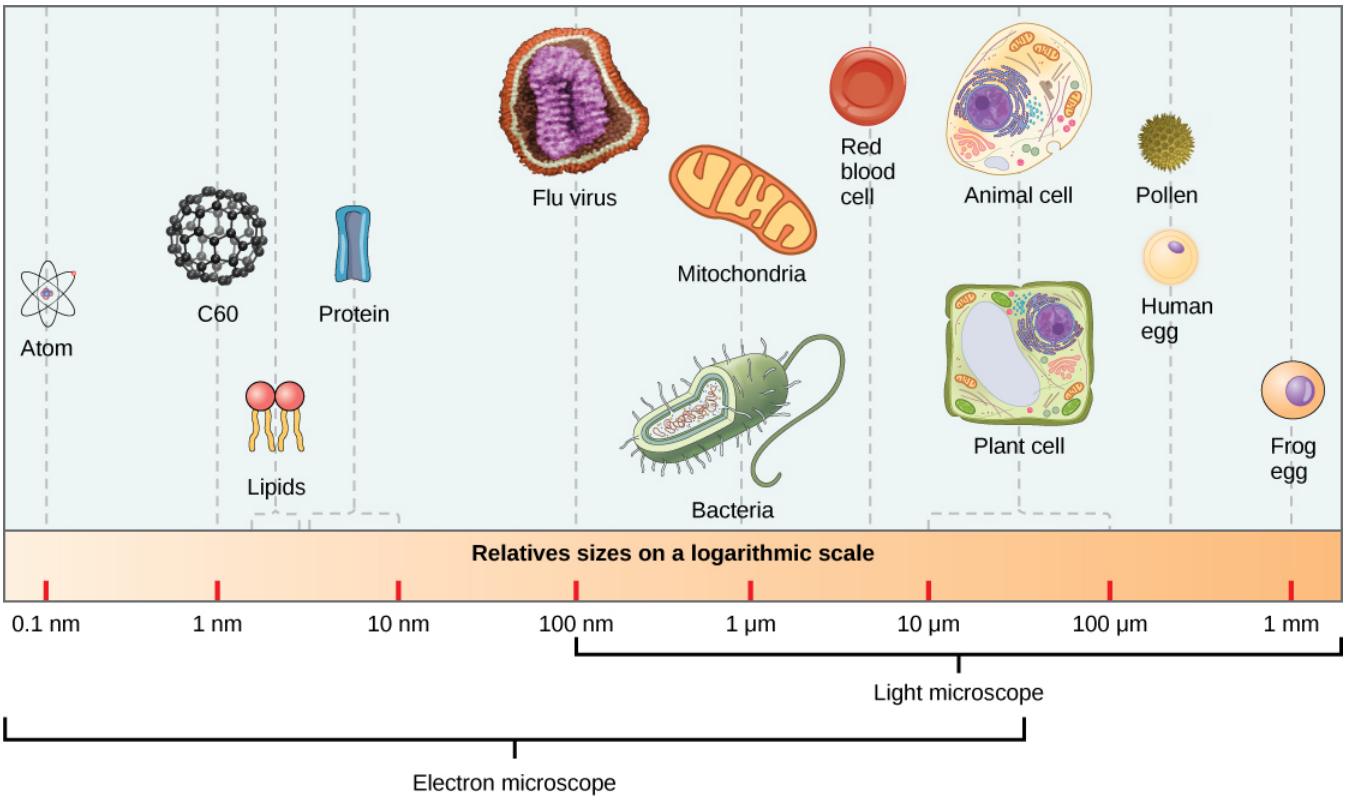


Figure 8.7.1.2: The size of a virus is very small relative to the size of cells and organelles.

It was not until the development of the electron microscope in the 1940s that scientists got their first good view of the structure of the tobacco mosaic virus (Figure **8.7.1.1**) and others. The surface structure of virions can be observed by both scanning and transmission electron microscopy, whereas the internal structures of the virus can only be observed in images from a transmission electron microscope (Figure **8.7.1.3**).

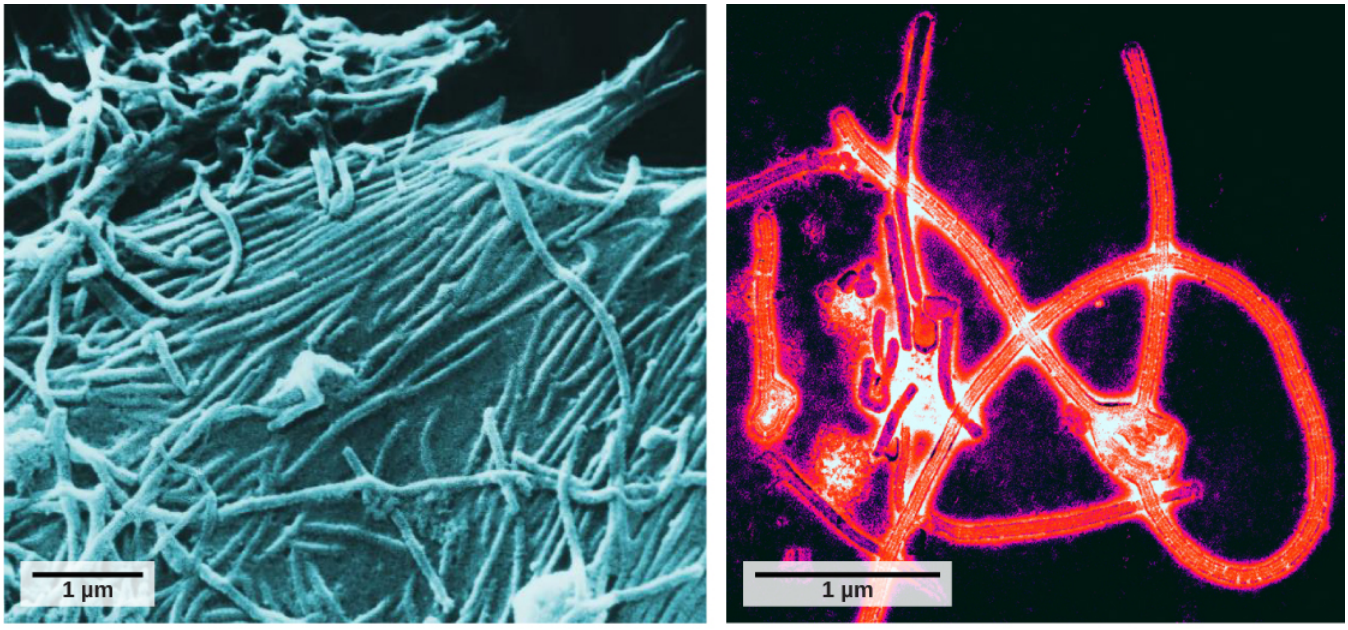


Figure 8.7.1.3: The ebola virus is shown here as visualized through (a) a scanning electron micrograph and (b) a transmission electron micrograph. (credit a: modification of work by Cynthia Goldsmith, CDC; credit b: modification of work by Thomas W. Geisbert, Boston University School of Medicine; scale-bar data from Matt Russell)

The use of this technology has allowed for the discovery of many viruses of all types of living organisms. They were initially grouped by shared morphology, meaning their size, shape, and distinguishing structures. Later, groups of viruses were classified by the type of nucleic acid they contained, DNA or RNA, and whether their nucleic acid was single- or double-stranded. More recently, molecular analysis of viral replication cycles has further refined their classification.

A virion consists of a nucleic-acid core, an outer protein coating, and sometimes an outer envelope made of protein and phospholipid membranes derived from the host cell. The most visible difference between members of viral families is their morphology, which is quite diverse. An interesting feature of viral complexity is that the complexity of the host does not correlate to the complexity of the virion. Some of the most complex virion structures are observed in bacteriophages, viruses that infect the simplest living organisms, bacteria.

Viruses come in many shapes and sizes, but these are consistent and distinct for each viral family (Figure **8.7.1.4**). All virions have a nucleic-acid genome covered by a protective layer of protein, called a capsid. The capsid is made of protein subunits called capsomeres. Some viral capsids are simple polyhedral “spheres,” whereas others are quite complex in structure. The outer structure surrounding the capsid of some viruses is called the viral envelope. All viruses use some sort of glycoprotein to attach to their host cells at molecules on the cell called viral receptors. The virus exploits these cell-surface molecules, which the cell uses for some other purpose, as a way to recognize and infect specific cell types. For example, the measles virus uses a cell-surface glycoprotein in humans that normally functions in immune reactions and possibly in the sperm-egg interaction at fertilization. Attachment is a requirement for viruses to later penetrate the cell membrane, inject the viral genome, and complete their replication inside the cell.

The T4 bacteriophage, which infects the *E. coli* bacterium, is among the most complex virion known; T4 has a protein tail structure that the virus uses to attach to the host cell and a head structure that houses its DNA.

Adenovirus, a nonenveloped animal virus that causes respiratory illnesses in humans, uses protein spikes protruding from its capsomeres to attach to the host cell. Nonenveloped viruses also include those that cause polio (poliovirus), plantar warts (papillomavirus), and hepatitis A (hepatitis A virus). Nonenveloped viruses tend to be more robust and more likely to survive under harsh conditions, such as the gut.

Enveloped virions like HIV (human immunodeficiency virus), the causative agent in AIDS (acquired immune deficiency syndrome), consist of nucleic acid (RNA in the case of HIV) and capsid proteins surrounded by a phospholipid bilayer envelope and its associated proteins (Figure **8.7.1.4**). Chicken pox, influenza, and mumps are examples of diseases caused by viruses with envelopes. Because of the fragility of the envelope, nonenveloped viruses are more resistant to changes in temperature, pH, and some disinfectants than enveloped viruses.

Overall, the shape of the virion and the presence or absence of an envelope tells us little about what diseases the viruses may cause or what species they might infect, but is still a useful means to begin viral classification.

ART CONNECTION

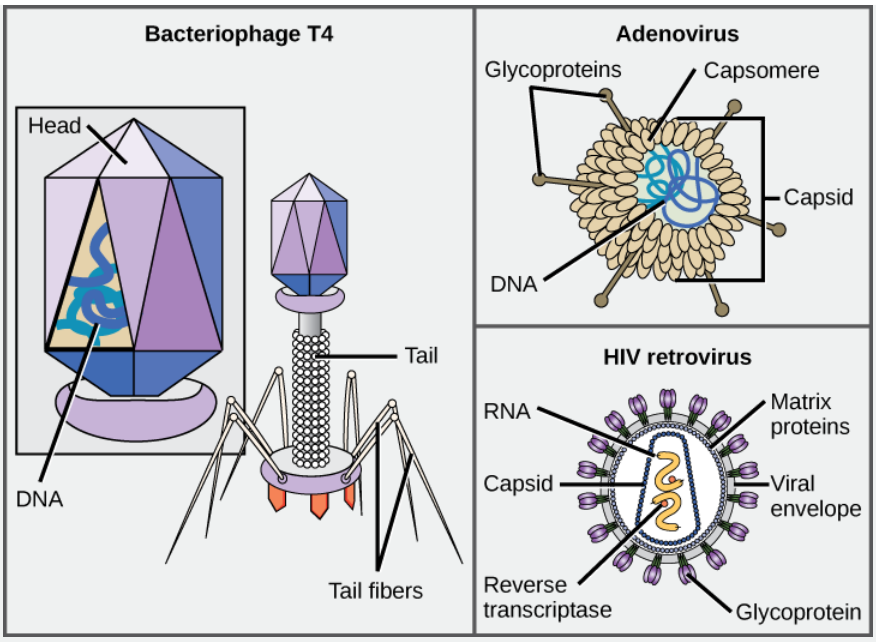


Figure 8.7.1.4: Viruses can be complex in shape or relatively simple. This figure shows three relatively complex virions: the bacteriophage T4, with its DNA-containing head group and tail fibers that attach to host cells; adenovirus, which uses spikes from its capsid to bind to the host cells; and HIV, which uses glycoproteins embedded in its envelope to do so. Notice that HIV has proteins called matrix proteins, internal to the envelope, which help stabilize virion shape. HIV is a retrovirus, which means it reverse transcribes its RNA genome into DNA, which is then spliced into the host's DNA. (credit "bacteriophage, adenovirus": modification of work by NCBI, NIH; credit "HIV retrovirus": modification of work by NIAID, NIH)

Which of the following statements about virus structure is true?

A. All viruses are encased in a viral membrane.
B. The capsomere is made up of small protein subunits called capsids.
C. DNA is the genetic material in all viruses.
D. Glycoproteins help the virus attach to the host cell.

Unlike all living organisms that use DNA as their genetic material, viruses may use either DNA or RNA as theirs. The virus core contains the genome or total genetic content of the virus. Viral genomes tend to be small compared to bacteria or eukaryotes, containing only those genes that code for proteins the virus cannot get from the host cell. This genetic material may be single-stranded or double-stranded. It may also be linear or circular. While most viruses contain a single segment of nucleic acid, others have genomes that consist of several segments.

DNA viruses have a DNA core. The viral DNA directs the host cell's replication proteins to synthesize new copies of the viral genome and to transcribe and translate that genome into viral proteins. DNA viruses cause human diseases such as chickenpox, hepatitis B, and some venereal diseases like herpes and genital warts.

RNA viruses contain only RNA in their cores. To replicate their genomes in the host cell, the genomes of RNA viruses encode enzymes not found in host cells. RNA polymerase enzymes are not as stable as DNA polymerases and often make mistakes during transcription. For this reason, mutations, changes in the nucleotide sequence, in RNA viruses occur more frequently than in DNA viruses. This leads to more rapid evolution and change in RNA viruses. For example, the fact that influenza is an RNA virus is one reason a new flu vaccine is needed every year. Human diseases caused by RNA viruses include hepatitis C, measles, and rabies.

Viruses can be seen as obligate intracellular parasites. The virus must attach to a living cell, be taken inside, manufacture its proteins and copy its genome, and find a way to escape the cell so the virus can infect other cells and ultimately other individuals. Viruses can infect only certain species of hosts and only certain cells within that host. The molecular basis for this specificity is that a particular surface molecule, known as the viral receptor, must be found on the host cell surface for the virus to attach. Also, metabolic differences seen in different cell types based on differential gene expression are a likely factor in which cells a virus may

use to replicate. The cell must be making the substances the virus needs, such as enzymes the virus genome itself does not have genes for, or the virus will not be able to replicate using that cell.

Steps of Virus Infections

A virus must “take over” a cell to replicate. The viral replication cycle can produce dramatic biochemical and structural changes in the host cell, which may cause cell damage. These changes, called cytopathic effects, can change cell functions or even destroy the cell. Some infected cells, such as those infected by the common cold virus (rhinovirus), die through lysis (bursting) or apoptosis (programmed cell death or “cell suicide”), releasing all the progeny virions at once. The symptoms of viral diseases result from the immune response to the virus, which attempts to control and eliminate the virus from the body, and from cell damage caused by the virus. Many animal viruses, such as HIV (human immunodeficiency virus), leave the infected cells of the immune system by a process known as budding, where virions leave the cell individually. During the budding process, the cell does not undergo lysis and is not immediately killed. However, the damage to the cells that HIV infects may make it impossible for the cells to function as mediators of immunity, even though the cells remain alive for a period of time. Most productive viral infections follow similar steps in the virus replication cycle: attachment, penetration, uncoating, replication, assembly, and release.

A virus attaches to a specific receptor site on the host-cell membrane through attachment proteins in the capsid or proteins embedded in its envelope. The attachment is specific, and typically a virus will only attach to cells of one or a few species and only certain cell types within those species with the appropriate receptors.

CONCEPT IN ACTION

View this video for a visual explanation of how influenza attacks the body.

Unlike animal viruses, the nucleic acid of bacteriophages is injected into the host cell naked, leaving the capsid outside the cell. Plant and animal viruses can enter their cells through endocytosis, in which the cell membrane surrounds and engulfs the entire virus. Some enveloped viruses enter the cell when the viral envelope fuses directly with the cell membrane. Once inside the cell, the viral capsid is degraded and the viral nucleic acid is released, which then becomes available for replication and transcription.

The replication mechanism depends on the viral genome. DNA viruses usually use host cell proteins and enzymes to make additional DNA that is used to copy the genome or be transcribed to messenger RNA (mRNA), which is then used in protein synthesis. RNA viruses, such as the influenza virus, usually use the RNA core as a template for synthesis of viral genomic RNA and mRNA. The viral mRNA is translated into viral enzymes and capsid proteins to assemble new virions (Figure 8.7.1.5). Of course, there are exceptions to this pattern. If a host cell does not provide the enzymes necessary for viral replication, viral genes supply the information to direct synthesis of the missing proteins. Retroviruses, such as HIV, have an RNA genome that must be reverse transcribed to make DNA, which then is inserted into the host’s DNA. To convert RNA into DNA, retroviruses contain genes that encode the virus-specific enzyme reverse transcriptase that transcribes an RNA template to DNA. The fact that HIV produces some of its own enzymes, which are not found in the host, has allowed researchers to develop drugs that inhibit these enzymes. These drugs, including the reverse transcriptase inhibitor AZT, inhibit HIV replication by reducing the activity of the enzyme without affecting the host’s metabolism.

The last stage of viral replication is the release of the new virions into the host organism, where they are able to infect adjacent cells and repeat the replication cycle. Some viruses are released when the host cell dies and other viruses can leave infected cells by budding through the membrane without directly killing the cell.

ART CONNECTION

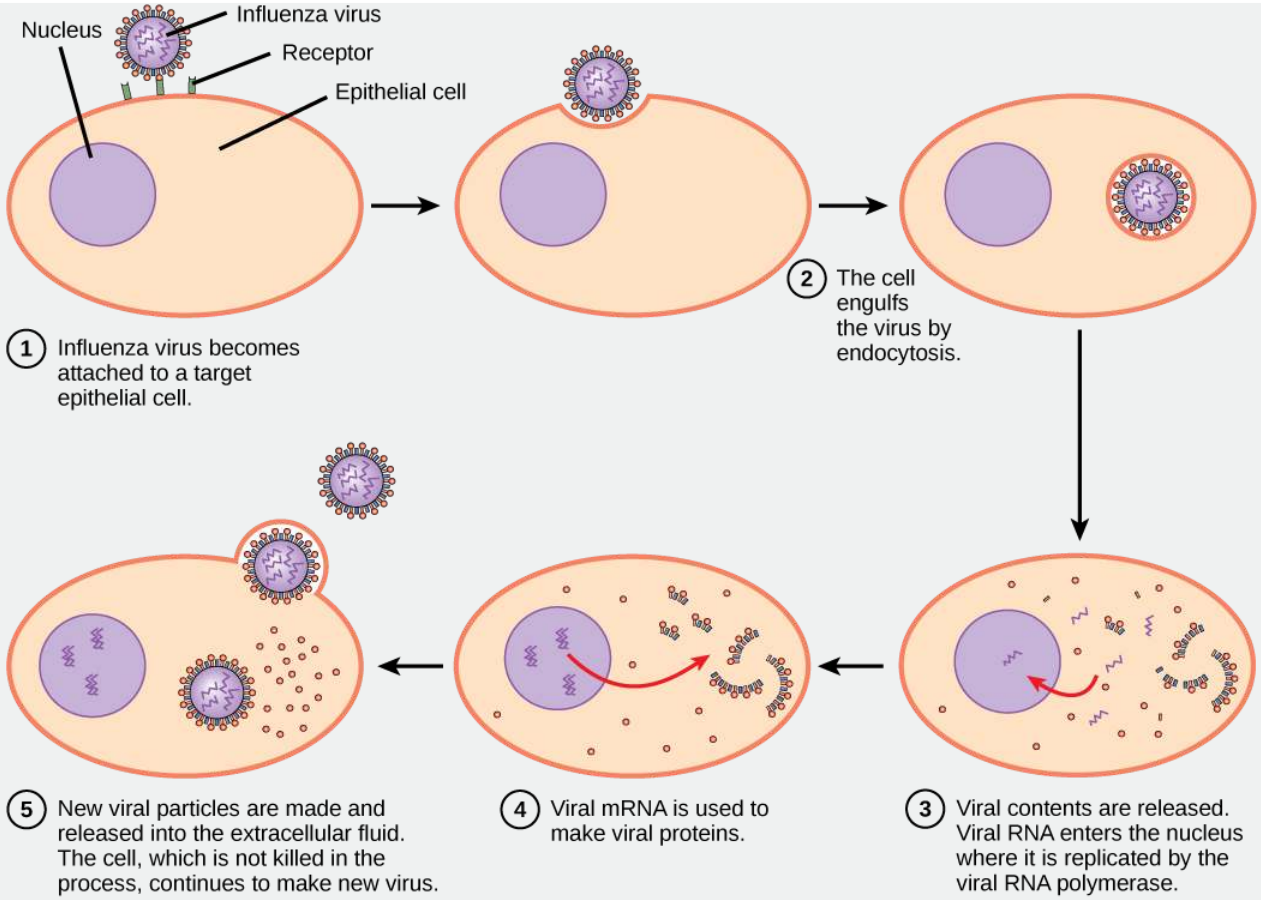


Figure 8.7.1.5: In influenza virus infection, glycoproteins attach to a host epithelial cell. As a result, the virus is engulfed. RNA and proteins are made and assembled into new virions.

Influenza virus is packaged in a viral envelope, which fuses with the plasma membrane. This way, the virus can exit the host cell without killing it. What advantage does the virus gain by keeping the host cell alive?

CONCEPT IN ACTION



Click through this tutorial on viruses to identify structures, modes of transmission, replication, and more.

Viruses and Disease

Viruses cause a variety of diseases in animals, including humans, ranging from the common cold to potentially fatal illnesses like meningitis (Figure 8.7.1.6). These diseases can be treated by antiviral drugs or by vaccines, but some viruses, such as HIV, are

capable of avoiding the immune response and mutating so as to become resistant to antiviral drugs.

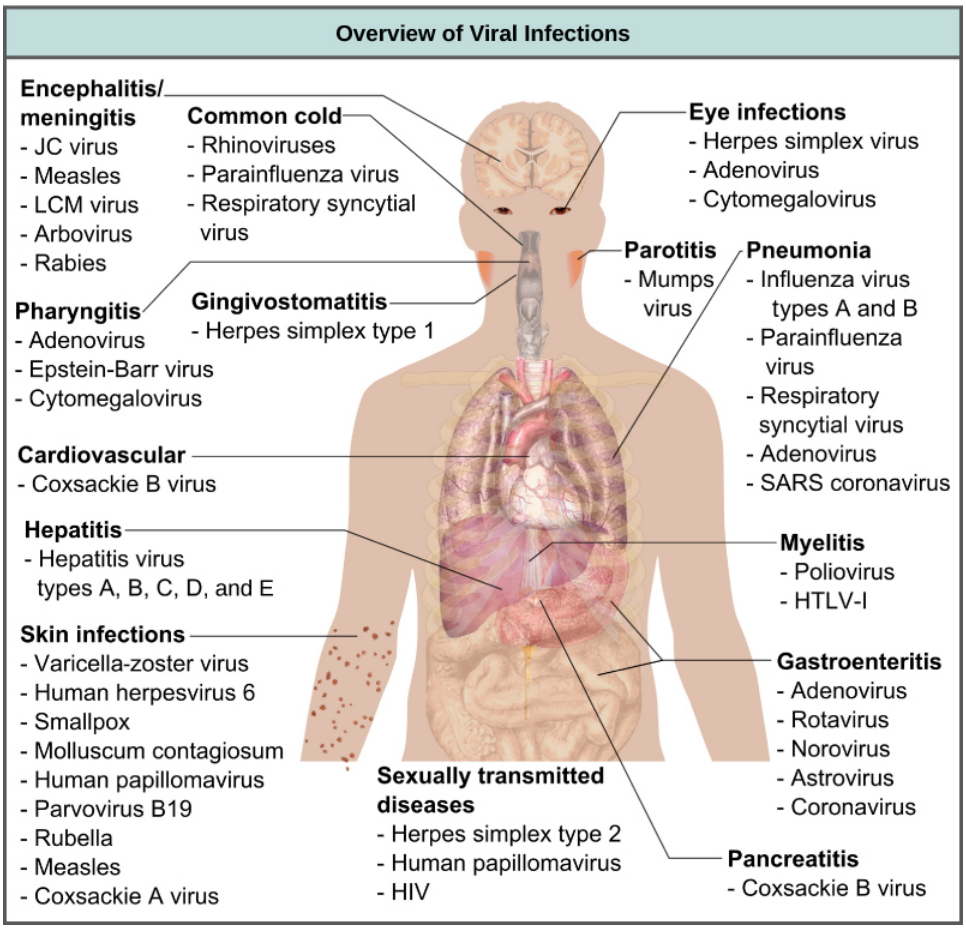


Figure 8.7.1.6: Viruses are the cause of dozens of ailments in humans, ranging from mild illnesses to serious diseases. (credit: modification of work by Mikael Häggström)

Vaccines for Prevention

While we do have limited numbers of effective antiviral drugs, such as those used to treat HIV and influenza, the primary method of controlling viral disease is by vaccination, which is intended to prevent outbreaks by building immunity to a virus or virus family. A vaccine may be prepared using weakened live viruses, killed viruses, or molecular subunits of the virus. In general, live viruses lead to better immunity, but have the possibility of causing disease at some low frequency. Killed viral vaccine and the subunit viruses are both incapable of causing disease, but in general lead to less effective or long-lasting immunity.

Weakened live viral vaccines are designed in the laboratory to cause few symptoms in recipients while giving them immunity against future infections. Polio was one disease that represented a milestone in the use of vaccines. Mass immunization campaigns in the U.S. in the 1950s (killed vaccine) and 1960s (live vaccine) essentially eradicated the disease, which caused muscle paralysis in children and generated fear in the general population when regional epidemics occurred. The success of the polio vaccine paved the way for the routine dispensation of childhood vaccines against measles, mumps, rubella, chickenpox, and other diseases.

Live vaccines are usually made by attenuation (weakening) of the “wild-type” (disease-causing) virus by growing it in the laboratory in tissues or at temperatures different from what the virus is accustomed to in the host. For example, the virus may be grown in cells in a test tube, in bird embryos, or in live animals. The adaptation to these new cells or temperature induces mutations in the virus’ genomes, allowing them to grow better in the laboratory while inhibiting their ability to cause disease when reintroduced into the conditions found in the host. These attenuated viruses thus still cause an infection, but they do not grow very well, allowing the immune response to develop in time to prevent major disease. The danger of using live vaccines, which are usually more effective than killed vaccines, is the low but significant risk that these viruses will revert back to their disease-causing form by back mutations. Back mutations occur when the vaccine undergoes mutations in the host such that it readapts to the host

and can again cause disease, which can then be spread to other humans in an epidemic. This happened as recently as 2007 in Nigeria where mutations in a polio vaccine led to an epidemic of polio in that country.

Some vaccines are in continuous development because certain viruses, such as influenza and HIV, have a high mutation rate compared to other viruses or host cells. With influenza, mutation in genes for the surface molecules helps the virus evade the protective immunity that may have been obtained in a previous influenza season, making it necessary for individuals to get vaccinated every year. Other viruses, such as those that cause the childhood diseases measles, mumps, and rubella, mutate so little that the same vaccine is used year after year.

Vaccines and Antiviral Drugs for Treatment

In some cases, vaccines can be used to treat an active viral infection. In the case of rabies, a fatal neurological disease transmitted in the saliva of rabies virus-infected animals, the progression of the disease from the time of the animal bite to the time it enters the central nervous system may be two weeks or longer. This is enough time to vaccinate an individual who suspects being bitten by a rabid animal, and the boosted immune response from the vaccination is enough to prevent the virus from entering nervous tissue. Thus, the fatal neurological consequences of the disease are averted and the individual only has to recover from the infected bite. This approach is also being used for the treatment of Ebola, one of the fastest and most deadly viruses affecting humans, though usually infecting limited populations. Ebola is also a leading cause of death in gorillas. Transmitted by bats and great apes, this virus can cause death in 70–90 percent of the infected within two weeks. Using newly developed vaccines that boost the immune response, there is hope that immune systems of affected individuals will be better able to control the virus, potentially reducing mortality rates.

Another way of treating viral infections is the use of antiviral drugs. These drugs often have limited ability to cure viral disease but have been used to control and reduce symptoms for a wide variety of viral diseases. For most viruses, these drugs inhibit the virus by blocking the actions of one or more of its proteins. It is important that the targeted proteins be encoded for by viral genes and that these molecules are not present in a healthy host cell. In this way, viral growth is inhibited without damaging the host. There are large numbers of antiviral drugs available to treat infections, some specific for a particular virus and others that can affect multiple viruses.

Antivirals have been developed to treat genital herpes (herpes simplex II) and influenza. For genital herpes, drugs such as acyclovir can reduce the number and duration of the episodes of active viral disease during which patients develop viral lesions in their skins cells. As the virus remains latent in nervous tissue of the body for life, this drug is not a cure but can make the symptoms of the disease more manageable. For influenza, drugs like Tamiflu can reduce the duration of “flu” symptoms by one or two days, but the drug does not prevent symptoms entirely. Other antiviral drugs, such as Ribavirin, have been used to treat a variety of viral infections.

By far the most successful use of antivirals has been in the treatment of the retrovirus HIV, which causes a disease that, if untreated, is usually fatal within 10–12 years after being infected. Anti-HIV drugs have been able to control viral replication to the point that individuals receiving these drugs survive for a significantly longer time than the untreated.

Anti-HIV drugs inhibit viral replication at many different phases of the HIV replicative cycle. Drugs have been developed that inhibit the fusion of the HIV viral envelope with the plasma membrane of the host cell (fusion inhibitors), the conversion of its RNA genome to double-stranded DNA (reverse transcriptase inhibitors), the integration of the viral DNA into the host genome (integrase inhibitors), and the processing of viral proteins (protease inhibitors).

When any of these drugs are used individually, the virus’ high mutation rate allows the virus to rapidly evolve resistance to the drug. The breakthrough in the treatment of HIV was the development of highly active anti-retroviral therapy (HAART), which involves a mixture of different drugs, sometimes called a drug “cocktail.” By attacking the virus at different stages of its replication cycle, it is difficult for the virus to develop resistance to multiple drugs at the same time. Still, even with the use of combination HAART therapy, there is concern that, over time, the virus will evolve resistance to this therapy. Thus, new anti-HIV drugs are constantly being developed with the hope of continuing the battle against this highly fatal virus.

Summary

Viruses are acellular entities that can usually only be seen with an electron microscope. Their genomes contain either DNA or RNA, and they replicate using the replication proteins of a host cell. Viruses are diverse, infecting archaea, bacteria, fungi, plants,

and animals. Viruses consist of a nucleic-acid core surrounded by a protein capsid with or without an outer lipid envelope.

Viral replication within a living cell always produces changes in the cell, sometimes resulting in cell death and sometimes slowly killing the infected cells. There are six basic stages in the virus replication cycle: attachment, penetration, uncoating, replication, assembly, and release. A viral infection may be productive, resulting in new virions, or nonproductive, meaning the virus remains inside the cell without producing new virions.

Viruses cause a variety of diseases in humans. Many of these diseases can be prevented by the use of viral vaccines, which stimulate protective immunity against the virus without causing major disease. Viral vaccines may also be used in active viral infections, boosting the ability of the immune system to control or destroy the virus. Antiviral drugs that target enzymes and other protein products of viral genes have been developed and used with mixed success. Combinations of anti-HIV drugs have been used to effectively control the virus, extending the lifespan of infected individuals.

Art Connections

Figure 8.7.1.4: Which of the following statements about virus structure is true?

A. All viruses are encased in a viral membrane.
B. The capsomere is made up of small protein subunits called capsids.
C. DNA is the genetic material in all viruses.
D. Glycoproteins help the virus attach to the host cell.

Answer

D

Figure 8.7.1.5: Influenza virus is packaged in a viral envelope, which fuses with the plasma membrane. This way, the virus can exit the host cell without killing it. What advantage does the virus gain by keeping the host cell alive?

Answer

The host cell can continue to make new virus particles.

Glossary

acellular
lacking cells

apoptosis
the cell death caused by induction of a cell's own internal mechanisms either as a natural step in the development of a multicellular organism or by other environmental factors such as signals from cells of the immune system

attenuation
the weakening of a virus during vaccine development

capsid
the protein coating of the viral core

cytopathic
causing cell damage

glycoprotein
a protein molecule with attached carbohydrate molecules

vaccine
a weakened solution of virus components, viruses, or other agents that produce an immune response

virion

an individual virus particle outside a host cell

viral envelope

a lipid bilayer that envelops some viruses

Contributors and Attributions

- Samantha Fowler (Clayton State University), Rebecca Roush (Sandhills Community College), James Wise (Hampton University). Original content by OpenStax (CC BY 4.0; Access for free at https://cnx.org/contents/b3c1e1d2-83...4-e119a8aafbdd).

This page titled 8.7.1: Viruses is shared under a CC BY license and was authored, remixed, and/or curated by .

- **17.1: Viruses** by OpenStax is licensed CC BY 4.0.

8.7.2: Innate Immunity

The vertebrate, including human, immune system is a complex multilayered system for defending against external and internal threats to the integrity of the body. The system can be divided into two types of defense systems: the innate immune system, which is nonspecific toward a particular kind of pathogen, and the adaptive immune system, which is specific (Figure 8.7.2.1). Innate immunity is not caused by an infection or vaccination and depends initially on physical and chemical barriers that work on all pathogens, sometimes called the first line of defense. The second line of defense of the innate system includes chemical signals that produce inflammation and fever responses as well as mobilizing protective cells and other chemical defenses. The adaptive immune system mounts a highly specific response to substances and organisms that do not belong in the body. The adaptive system takes longer to respond and has a memory system that allows it to respond with greater intensity should the body reencounter a pathogen even years later.

Vertebrate Immunity		
Innate Immune System		Adaptive Immune System
Physical Barriers	Internal Defenses	
• Skin, hair, cilia • Mucus membranes • Mucus and chemical secretions • Digestive enzymes in mouth • Stomach acid	• Inflammatory response • Complement proteins • Phagocytic cells • Natural killer (NK) cells	• Antibodies and the humoral immune response • Cell-mediated immune response • Memory response

Figure 8.7.2.1: There are two main parts to the vertebrate immune system. The innate immune system, which is made up of physical barriers and internal defenses, responds to all pathogens. The adaptive immune system is highly specific.

External and Chemical Barriers

The body has significant physical barriers to potential pathogens. The skin contains the protein keratin, which resists physical entry into cells. Other body surfaces, particularly those associated with body openings, are protected by the mucous membranes. The sticky mucus provides a physical trap for pathogens, preventing their movement deeper into the body. The openings of the body, such as the nose and ears, are protected by hairs that catch pathogens, and the mucous membranes of the upper respiratory tract have cilia that constantly move pathogens trapped in the mucus coat up to the mouth.

The skin and mucous membranes also create a chemical environment that is hostile to many microorganisms. The surface of the skin is acidic, which prevents bacterial growth. Saliva, mucus, and the tears of the eye contain an enzyme that breaks down bacterial cell walls. The stomach secretions create a highly acidic environment, which kills many pathogens entering the digestive system.

Finally, the surface of the body and the lower digestive system have a community of microorganisms such as bacteria, archaea, and fungi that coexist without harming the body. There is evidence that these organisms are highly beneficial to their host, combating disease-causing organisms and outcompeting them for nutritional resources provided by the host body. Despite these defenses, pathogens may enter the body through skin abrasions or punctures, or by collecting on mucosal surfaces in large numbers that overcome the protections of mucus or cilia.

Internal Defenses

When pathogens enter the body, the innate immune system responds with a variety of internal defenses. These include the inflammatory response, phagocytosis, natural killer cells, and the complement system. White blood cells in the blood and lymph recognize pathogens as foreign to the body. A white blood cell is larger than a red blood cell, is nucleated, and is typically able to move using amoeboid locomotion. Because they can move on their own, white blood cells can leave the blood to go to infected tissues. For example, a monocyte is a type of white blood cell that circulates in the blood and lymph and develops into a

macrophage after it moves into infected tissue. A macrophage is a large cell that engulfs foreign particles and pathogens. Mast cells are produced in the same way as white blood cells, but unlike circulating white blood cells, mast cells take up residence in connective tissues and especially mucosal tissues. They are responsible for releasing chemicals in response to physical injury. They also play a role in the allergic response, which will be discussed later in the chapter.

When a pathogen is recognized as foreign, chemicals called cytokines are released. A cytokine is a chemical messenger that regulates cell differentiation (form and function), proliferation (production), and gene expression to produce a variety of immune responses. Approximately 40 types of cytokines exist in humans. In addition to being released from white blood cells after pathogen recognition, cytokines are also released by the infected cells and bind to nearby uninfected cells, inducing those cells to release cytokines. This positive feedback loop results in a burst of cytokine production.

One class of early-acting cytokines is the interferons, which are released by infected cells as a warning to nearby uninfected cells. An interferon is a small protein that signals a viral infection to other cells. The interferons stimulate uninfected cells to produce compounds that interfere with viral replication. Interferons also activate macrophages and other cells.

The Inflammatory Response and Phagocytosis

The first cytokines to be produced encourage inflammation, a localized redness, swelling, heat, and pain. Inflammation is a response to physical trauma, such as a cut or a blow, chemical irritation, and infection by pathogens (viruses, bacteria, or fungi). The chemical signals that trigger an inflammatory response enter the extracellular fluid and cause capillaries to dilate (expand) and capillary walls to become more permeable, or leaky. The serum and other compounds leaking from capillaries cause swelling of the area, which in turn causes pain. Various kinds of white blood cells are attracted to the area of inflammation. The types of white blood cells that arrive at an inflamed site depend on the nature of the injury or infecting pathogen. For example, a neutrophil is an early arriving white blood cell that engulfs and digests pathogens. Neutrophils are the most abundant white blood cells of the immune system (Figure **8.7.2.2**). Macrophages follow neutrophils and take over the phagocytosis function and are involved in the resolution of an inflamed site, cleaning up cell debris and pathogens.

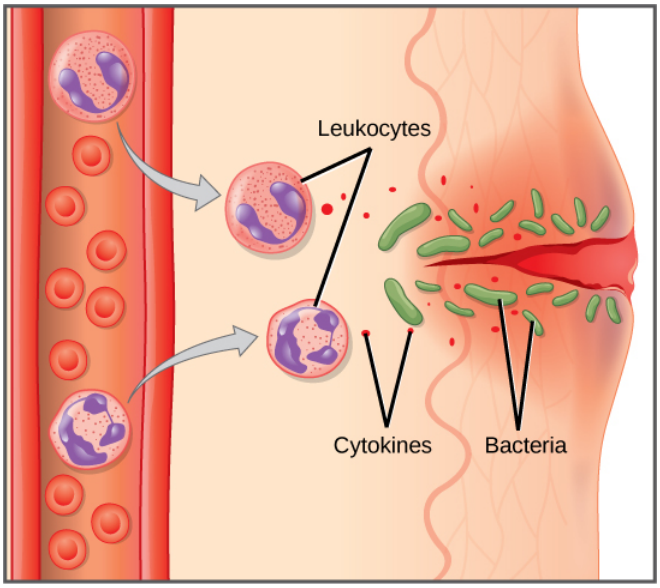


Figure 8.7.2.2: White blood cells (leukocytes) release chemicals to stimulate the inflammatory response following a cut in the skin.

Cytokines also send feedback to cells of the nervous system to bring about the overall symptoms of feeling sick, which include lethargy, muscle pain, and nausea. Cytokines also increase the core body temperature, causing a fever. The elevated temperatures of a fever inhibit the growth of pathogens and speed up cellular repair processes. For these reasons, suppression of fevers should be limited to those that are dangerously high.

CONCEPT IN ACTION

Check out this 23-second, stop-motion video showing a neutrophil that searches and engulfs fungus spores during an elapsed time of 79 minutes.

Natural Killer Cells

A lymphocyte is a white blood cell that contains a large nucleus (Figure 8.7.2.3). Most lymphocytes are associated with the adaptive immune response, but infected cells are identified and destroyed by natural killer cells, the only lymphocytes of the innate immune system. A natural killer (NK) cell is a lymphocyte that can kill cells infected with viruses (or cancerous cells). NK cells identify intracellular infections, especially from viruses, by the altered expression of major histocompatibility class (MHC) I molecules on the surface of infected cells. MHC class I molecules are proteins on the surfaces of all nucleated cells that provide a sample of the cell's internal environment at any given time. Unhealthy cells, whether infected or cancerous, display an altered MHC class I complement on their cell surfaces.

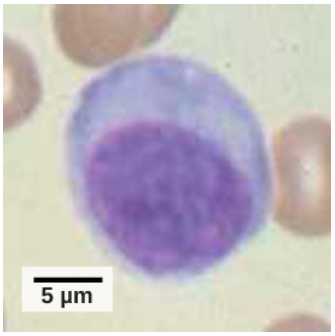


Figure 8.7.2.3: Lymphocytes, such as NK cells, are characterized by their large nuclei that actively absorb Wright stain and therefore appear dark colored under a microscope. (credit: scale-bar data from Matt Russell)

After the NK cell detects an infected or tumor cell, it induces programmed cell death, or apoptosis. Phagocytic cells then come along and digest the cell debris left behind. NK cells are constantly patrolling the body and are an effective mechanism for controlling potential infections and preventing cancer progression. The various types of immune cells are shown in Figure 8.7.2.4.

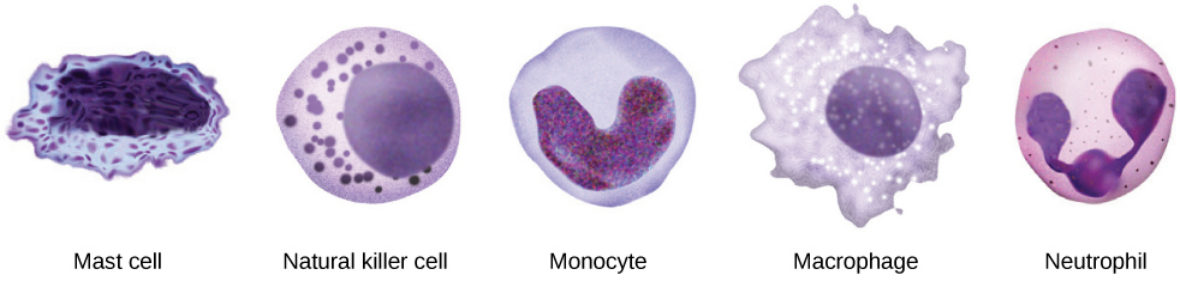


Figure 8.7.2.4: Cells involved in the innate immune response include mast cells, natural killer cells, and white blood cells, such as monocytes, macrophages and neutrophils.

Complement

An array of approximately 20 types of proteins, called a complement system, is also activated by infection or the activity of the cells of the adaptive immune system and functions to destroy extracellular pathogens. Liver cells and macrophages synthesize inactive forms of complement proteins continuously; these proteins are abundant in the blood serum and are capable of responding immediately to infecting microorganisms. The complement system is so named because it is complementary to the innate and adaptive immune system. Complement proteins bind to the surfaces of microorganisms and are particularly attracted to pathogens that are already tagged by the adaptive immune system. This "tagging" involves the attachment of specific proteins called antibodies (discussed in detail later) to the pathogen. When they attach, the antibodies change shape providing a binding site for one of the complement proteins. After the first few complement proteins bind, a cascade of binding in a specific sequence of proteins follows in which the pathogen rapidly becomes coated in complement proteins.

Complement proteins perform several functions, one of which is to serve as a marker to indicate the presence of a pathogen to phagocytic cells and enhance engulfment. Certain complement proteins can combine to open pores in microbial cell membranes

and cause lysis of the cells.

Summary

The innate immune system consists first of physical and chemical barriers to infection including the skin and mucous membranes and their secretions, ciliated surfaces, and body hairs. The second line of defense is an internal defense system designed to counter pathogenic threats that bypass the physical and chemical barriers of the body. Using a combination of cellular and molecular responses, the innate immune system identifies the nature of a pathogen and responds with inflammation, phagocytosis, cytokine release, destruction by NK cells, or the complement system.

Glossary

complement system
an array of approximately 20 soluble proteins of the innate immune system that enhance phagocytosis, bore holes in pathogens, and recruit lymphocytes

cytokine
a chemical messenger that regulates cell differentiation, proliferation, and gene expression to effect immune responses

inflammation
the localized redness, swelling, heat, and pain that results from the movement of leukocytes through opened capillaries to a site of infection

innate immunity
an immunity that occurs naturally because of genetic factors or physiology, and is not caused by infection or vaccination

interferon
a cytokine that inhibits viral replication

lymphocyte
a type of white blood cell that includes natural killer cells of the innate immune system and B and T cells of the adaptive immune system

macrophage
a large phagocytic cell that engulfs foreign particles and pathogens

major histocompatibility class (MHC) I
a group of proteins found on the surface of all nucleated cells that signals to immune cells whether the cell is normal or is infected or cancerous; it also provides the appropriate sites into which antigens can be loaded for recognition by lymphocytes

mast cell
a leukocyte that produces inflammatory molecules, such as histamine, in response to large pathogens

monocyte
a type of white blood cell that circulates in the blood and lymph and differentiates into a macrophage after it moves into infected tissue

natural killer (NK) cell
a lymphocyte that can kill cells infected with viruses or tumor cells

neutrophil
a phagocytic leukocyte that engulfs and digests pathogens

white blood cell

a nucleated cell found in the blood that is a part of the immune system; also called leukocytes

Contributors and Attributions

- Samantha Fowler (Clayton State University), Rebecca Roush (Sandhills Community College), James Wise (Hampton University). Original content by OpenStax (CC BY 4.0; Access for free at https://cnx.org/contents/b3c1e1d2-83...4-e119a8aafbdd).

This page titled 8.7.2: Innate Immunity is shared under a CC BY license and was authored, remixed, and/or curated by .

- **17.2: Innate Immunity** by OpenStax is licensed CC BY 4.0.

8.7.3: Adaptive Immunity

The adaptive, or acquired, immune response takes days or even weeks to become established—much longer than the innate response; however, adaptive immunity is more specific to an invading pathogen. Adaptive immunity is an immunity that occurs after exposure to an antigen either from a pathogen or a vaccination. An antigen is a molecule that stimulates a response in the immune system. This part of the immune system is activated when the innate immune response is insufficient to control an infection. In fact, without information from the innate immune system, the adaptive response could not be mobilized. There are two types of adaptive responses: the cell-mediated immune response, which is controlled by activated T cells, and the humoral immune response, which is controlled by activated B cells and antibodies. Activated T and B cells whose surface binding sites are specific to the molecules on the pathogen greatly increase in numbers and attack the invading pathogen. Their attack can kill pathogens directly or they can secrete antibodies that enhance the phagocytosis of pathogens and disrupt the infection. Adaptive immunity also involves a memory to give the host long-term protection from reinfection with the same type of pathogen; on reexposure, this host memory will facilitate a rapid and powerful response.

B and T Cells

Lymphocytes, which are white blood cells, are formed with other blood cells in the red bone marrow found in many flat bones, such as the shoulder or pelvic bones. The two types of lymphocytes of the adaptive immune response are B and T cells (Figure 8.7.3.1). Whether an immature lymphocyte becomes a B cell or T cell depends on where in the body it matures. The B cells remain in the bone marrow to mature (hence the name “B” for “bone marrow”), while T cells migrate to the thymus, where they mature (hence the name “T” for “thymus”).

Maturation of a B or T cell involves becoming immunocompetent, meaning that it can recognize, by binding, a specific molecule or antigen (discussed below). During the maturation process, B and T cells that bind too strongly to the body’s own cells are eliminated in order to minimize an immune response against the body’s own tissues. Those cells that react weakly to the body’s own cells, but have highly specific receptors on their cell surfaces that allow them to recognize a foreign molecule, or antigen, remain. This process occurs during fetal development and continues throughout life. The specificity of this receptor is determined by the genetics of the individual and is present before a foreign molecule is introduced to the body or encountered. Thus, it is genetics and not experience that initially provides a vast array of cells, each capable of binding to a different specific foreign molecule. Once they are immunocompetent, the T and B cells will migrate to the spleen and lymph nodes where they will remain until they are called on during an infection. B cells are involved in the humoral immune response, which targets pathogens loose in blood and lymph, and T cells are involved in the cell-mediated immune response, which targets infected cells.

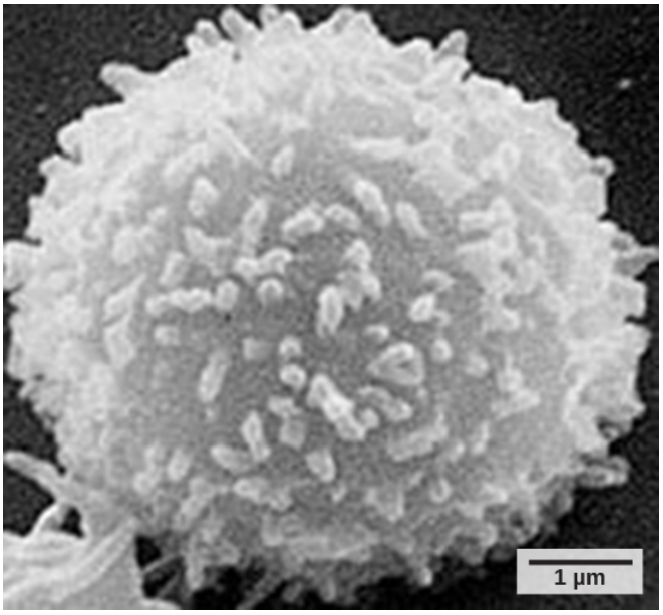


Figure 8.7.3.1: This scanning electron micrograph shows a T lymphocyte. T and B cells are indistinguishable by light microscopy but can be differentiated experimentally by probing their surface receptors. (credit: modification of work by NCI; scale-bar data from Matt Russell)

Humoral Immune Response

As mentioned, an antigen is a molecule that stimulates a response in the immune system. Not every molecule is antigenic. B cells participate in a chemical response to antigens present in the body by producing specific antibodies that circulate throughout the body and bind with the antigen whenever it is encountered. This is known as the humoral immune response. As discussed, during maturation of B cells, a set of highly specific B cells are produced that have many antigen receptor molecules in their membrane (Figure 8.7.3.2).

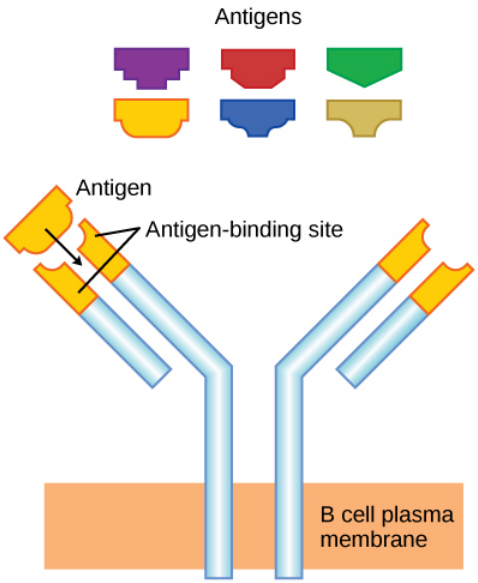


Figure 8.7.3.2: B cell receptors are embedded in the membranes of B cells and bind a variety of antigens through their variable regions.

Each B cell has only one kind of antigen receptor, which makes every B cell different. Once the B cells mature in the bone marrow, they migrate to lymph nodes or other lymphatic organs. When a B cell encounters the antigen that binds to its receptor, the antigen molecule is brought into the cell by endocytosis and reappears on the surface of the cell bound to an MHC class II molecule. When this process is complete, the B cell is sensitized. In most cases, the sensitized B cell must then encounter a specific kind of T cell, called a helper T cell, before it is activated. The helper T cell must already have been activated through an encounter with the antigen (discussed below).

The helper T cell binds to the antigen-MHC class II complex and is induced to release cytokines that induce the B cell to divide rapidly, which makes thousands of identical (clonal) cells. These daughter cells become either plasma cells or memory B cells. The memory B cells remain inactive at this point, until another later encounter with the antigen, caused by a reinfection by the same bacteria or virus, results in them dividing into a new population of plasma cells. The plasma cells, on the other hand, produce and secrete large quantities, up to 100 million molecules per hour, of antibody molecules. An antibody, also known as an immunoglobulin (Ig), is a protein that is produced by plasma cells after stimulation by an antigen. Antibodies are the agents of humoral immunity. Antibodies occur in the blood, in gastric and mucus secretions, and in breast milk. Antibodies in these bodily fluids can bind pathogens and mark them for destruction by phagocytes before they can infect cells.

These antibodies circulate in the blood stream and lymphatic system and bind with the antigen whenever it is encountered. The binding can fight infection in several ways. Antibodies can bind to viruses or bacteria and interfere with the chemical interactions required for them to infect or bind to other cells. The antibodies may create bridges between different particles containing antigenic sites clumping them all together and preventing their proper functioning. The antigen-antibody complex stimulates the complement system described previously, destroying the cell bearing the antigen. Phagocytic cells, such as those already described, are attracted by the antigen-antibody complexes, and phagocytosis is enhanced when the complexes are present. Finally, antibodies stimulate inflammation, and their presence in mucus and on the skin prevents pathogen attack.

Antibodies coat extracellular pathogens and neutralize them by blocking key sites on the pathogen that enhance their infectivity (such as receptors that “dock” pathogens on host cells) (Figure 8.7.3.3). Antibody neutralization can prevent pathogens from

entering and infecting host cells. The neutralized antibody-coated pathogens can then be filtered by the spleen and eliminated in urine or feces.

Antibodies also mark pathogens for destruction by phagocytic cells, such as macrophages or neutrophils, in a process called opsonization. In a process called complement fixation, some antibodies provide a place for complement proteins to bind. The combination of antibodies and complement promotes rapid clearing of pathogens.

The production of antibodies by plasma cells in response to an antigen is called active immunity and describes the host's active response of the immune system to an infection or to a vaccination. There is also a passive immune response where antibodies come from an outside source, instead of the individual's own plasma cells, and are introduced into the host. For example, antibodies circulating in a pregnant woman's body move across the placenta into the developing fetus. The child benefits from the presence of these antibodies for up to several months after birth. In addition, a passive immune response is possible by injecting antibodies into an individual in the form of an antivenom to a snake-bite toxin or antibodies in blood serum to help fight a hepatitis infection. This gives immediate protection since the body does not need the time required to mount its own response.

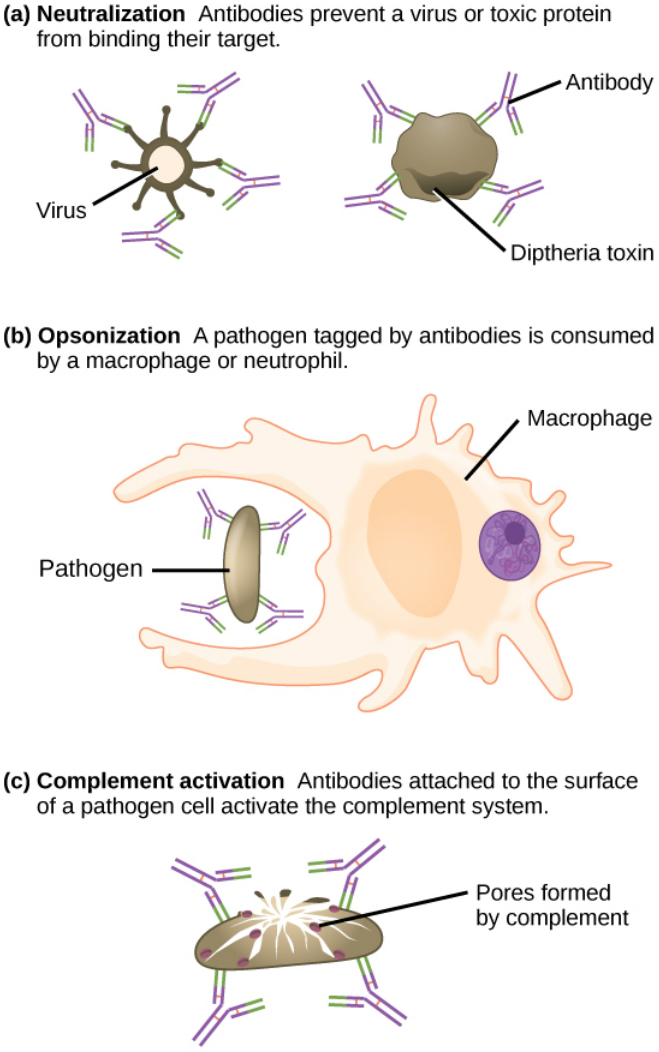


Figure 8.7.3.3: Antibodies may inhibit infection by (a) preventing the antigen from binding its target, (b) tagging a pathogen for destruction by macrophages or neutrophils, or (c) activating the complement cascade.

Cell-Mediated Immunity

Unlike B cells, T lymphocytes are unable to recognize pathogens without assistance. Instead, dendritic cells and macrophages first engulf and digest pathogens into hundreds or thousands of antigens. Then, an antigen-presenting cell (APC) detects, engulfs, and informs the adaptive immune response about an infection. When a pathogen is detected, these APCs will engulf and break it down

through phagocytosis. Antigen fragments will then be transported to the surface of the APC, where they will serve as an indicator to other immune cells. A dendritic cell is an immune cell that mops up antigenic materials in its surroundings and presents them on its surface. Dendritic cells are located in the skin, the linings of the nose, lungs, stomach, and intestines. These positions are ideal locations to encounter invading pathogens. Once they are activated by pathogens and mature to become APCs they migrate to the spleen or a lymph node. Macrophages also function as APCs. After phagocytosis by a macrophage, the phagocytic vesicle fuses with an intracellular lysosome. Within the resulting phagolysosome, the components are broken down into fragments; the fragments are then loaded onto MHC class II molecules and are transported to the cell surface for antigen presentation (Figure 8.7.3.4). Helper T cells cannot properly respond to an antigen unless it is processed and embedded in an MHC class II molecule. The APCs express MHC class II on their surfaces, and when combined with a foreign antigen, these complexes signal an invader.

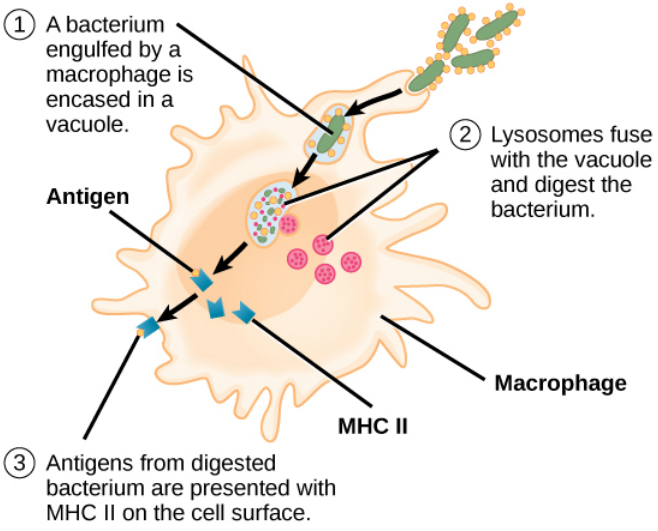


Figure 8.7.3.4: An antigen-presenting cell (APC), such as a macrophage, engulfs a foreign antigen, partially digests it in a lysosome, and then embeds it in an MHC class II molecule for presentation at the cell surface. Lymphocytes of the adaptive immune response must interact with antigen-embedded MHC class II molecules to mature into functional immune cells.

T cells have many functions. Some respond to APCs of the innate immune system and indirectly induce immune responses by releasing cytokines. Others stimulate B cells to start the humoral response as described previously. Another type of T cell detects APC signals and directly kills the infected cells, while some are involved in suppressing inappropriate immune reactions to harmless or “self” antigens.

There are two main types of T cells: helper T lymphocytes (T_H) and the cytotoxic T lymphocytes (T_C). The T_H lymphocytes function indirectly to tell other immune cells about potential pathogens. T_H lymphocytes recognize specific antigens presented by the MHC class II complexes of APCs. There are two populations of T_H cells: T_H1 and T_H2. T_H1 cells secrete cytokines to enhance the activities of macrophages and other T cells. T_H2 cells stimulate naïve B cells to secrete antibodies. Whether a T_H1 or a T_H2 immune response develops depends on the specific types of cytokines secreted by cells of the innate immune system, which in turn depends on the nature of the invading pathogen.

Cytotoxic T cells (T_C) are the key component of the cell-mediated part of the adaptive immune system and attack and destroy infected cells. T_C cells are particularly important in protecting against viral infections; this is because viruses replicate within cells where they are shielded from extracellular contact with circulating antibodies. Once activated, the T_C creates a large clone of cells with one specific set of cell-surface receptors, as in the case with proliferation of activated B cells. As with B cells, the clone includes active T_C cells and inactive memory $T_C$cells. The resulting active T_C cells then identify infected host cells. Because of the time required to generate a population of clonal T and B cells, there is a delay in the adaptive immune response compared to the innate immune response.

T_C cells attempt to identify and destroy infected cells before the pathogen can replicate and escape, thereby halting the progression of intracellular infections. T_C cells also support NK lymphocytes to destroy early cancers. Cytokines secreted by the T_H1 response that stimulates macrophages also stimulate T_C cells and enhance their ability to identify and destroy infected cells and tumors. A summary of how the humoral and cell-mediated immune responses are activated appears in Figure 8.7.3.5.

B plasma cells and T_C cells are collectively called effector cells because they are involved in “effecting” (bringing about) the immune response of killing pathogens and infected host cells.

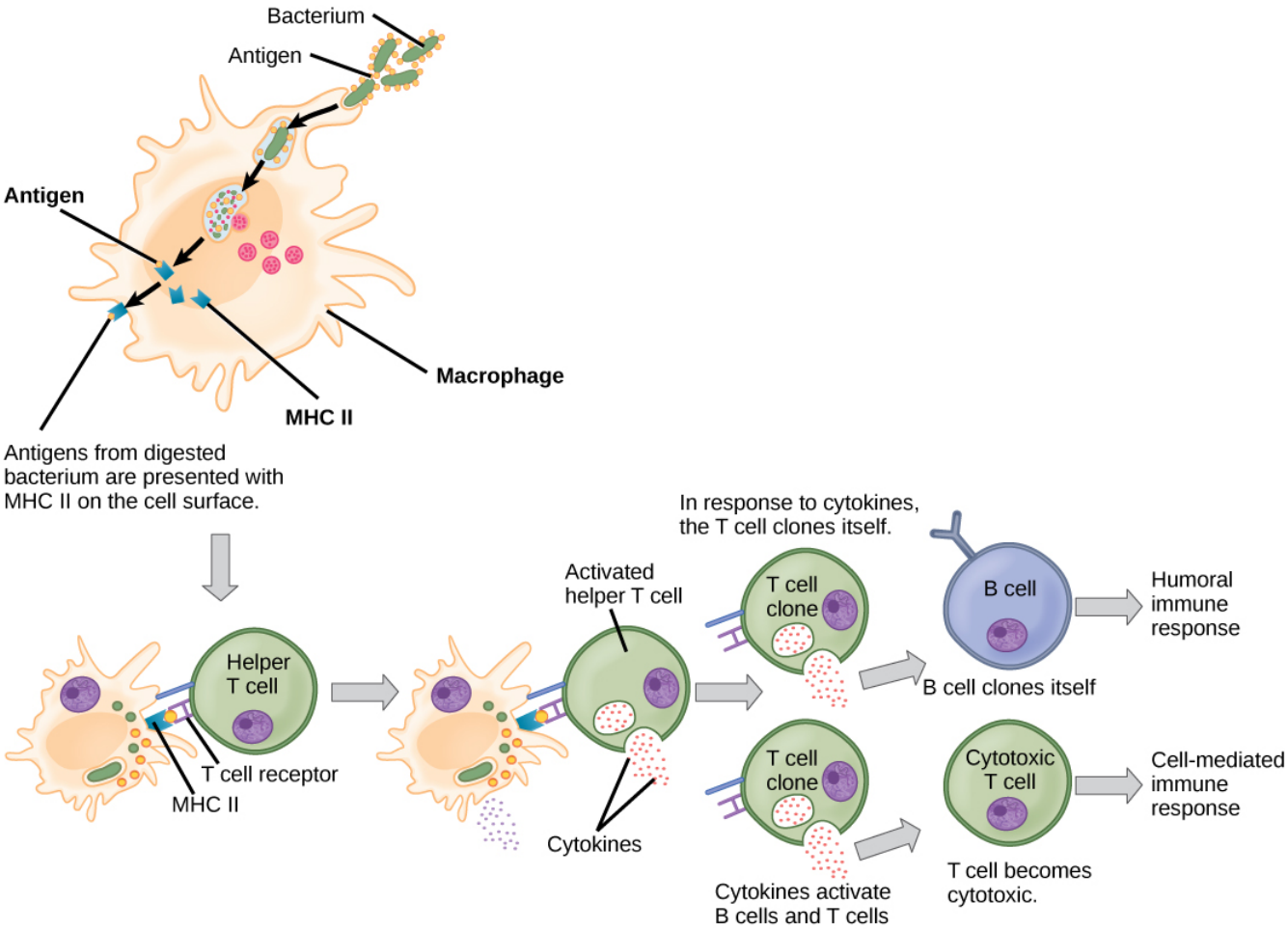


Figure 8.7.3.5: A helper T cell becomes activated by binding to an antigen presented by an APC via the MHCII receptor, causing it to release cytokines. Depending on the cytokines released, this activates either the humoral or the cell-mediated immune response.

Immunological Memory

The adaptive immune system has a memory component that allows for a rapid and large response upon reinvasion of the same pathogen. During the adaptive immune response to a pathogen that has not been encountered before, known as the primary immune response, plasma cells secreting antibodies and differentiated T cells increase, then plateau over time. As B and T cells mature into effector cells, a subset of the naïve populations differentiates into B and T memory cells with the same antigen specificities (Figure 8.7.3.6). A memory cell is an antigen-specific B or T lymphocyte that does not differentiate into an effector cell during the primary immune response, but that can immediately become an effector cell on reexposure to the same pathogen. As the infection is cleared and pathogenic stimuli subside, the effectors are no longer needed and they undergo apoptosis. In contrast, the memory cells persist in the circulation.

ART CONNECTION

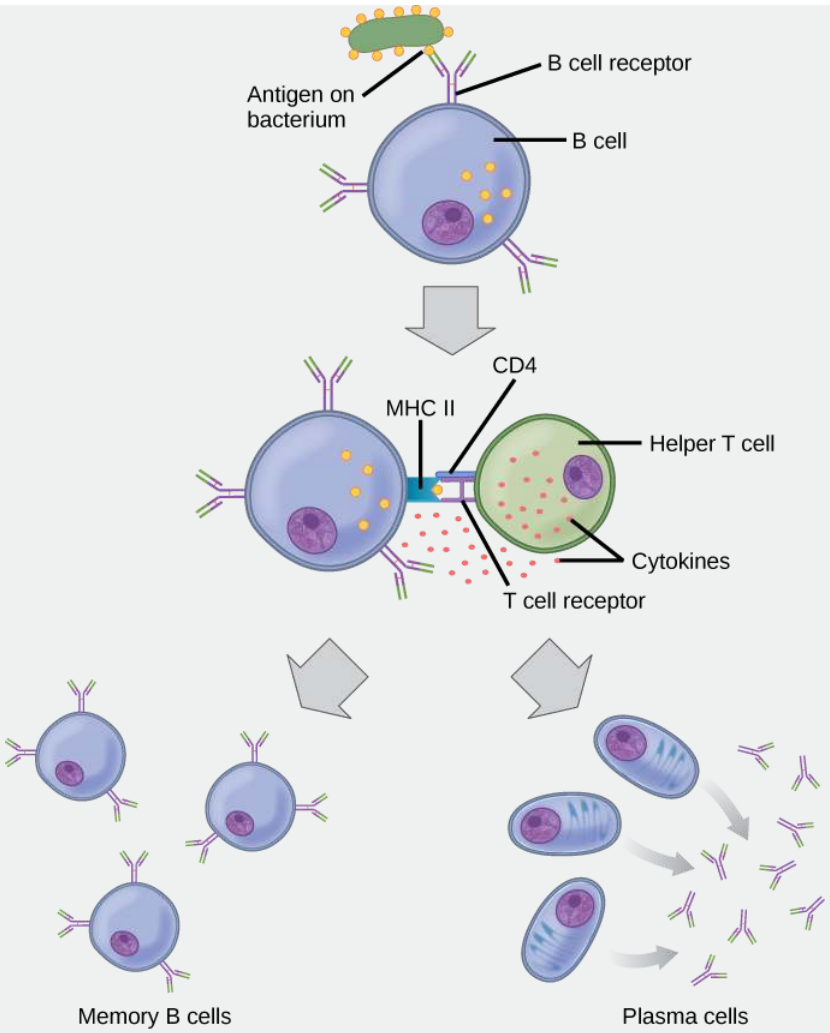


Figure 8.7.3.6: After initially binding an antigen to the B cell receptor, a B cell internalizes the antigen and presents it on MHC class II. A helper T cell recognizes the MHC class II- antigen complex and activates the B cell. As a result, memory B cells and plasma cells are made.

The Rh antigen is found on Rh-positive red blood cells. An Rh-negative female can usually carry an Rh-positive fetus to term without difficulty. However, if she has a second Rh-positive fetus, her body may launch an immune attack that causes hemolytic disease of the newborn. Why do you think hemolytic disease is only a problem during the second or subsequent pregnancies?

If the pathogen is never encountered again during the individual's lifetime, B and T memory cells will circulate for a few years or even several decades and will gradually die off, having never functioned as effector cells. However, if the host is re-exposed to the same pathogen type, circulating memory cells will immediately differentiate into plasma cells and T_C cells without input from APCs or T_H cells. This is known as the secondary immune response. One reason why the adaptive immune response is delayed is because it takes time for naïve B and T cells with the appropriate antigen specificities to be identified, activated, and proliferate. On reinfection, this step is skipped, and the result is a more rapid production of immune defenses. Memory B cells that differentiate into plasma cells output tens to hundreds-fold greater antibody amounts than were secreted during the primary response (Figure 8.7.3.7). This rapid and dramatic antibody response may stop the infection before it can even become established, and the individual may not realize they had been exposed.

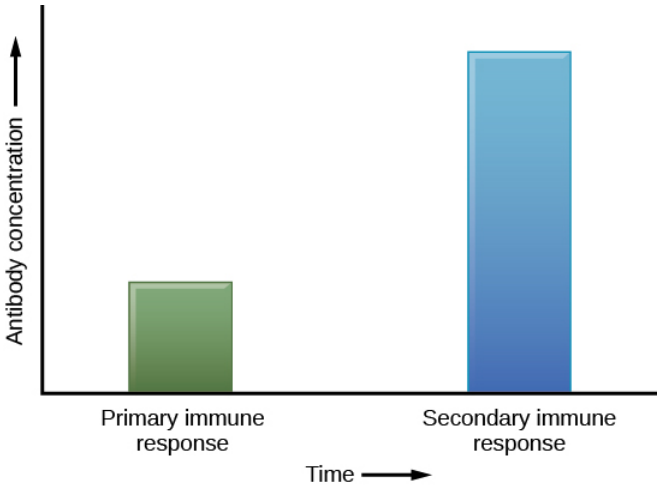


Figure 8.7.3.7: In the primary response to infection, antibodies are secreted first from plasma cells. Upon re-exposure to the same pathogen, memory cells differentiate into antibody-secreting plasma cells that output a greater amount of antibody for a longer period of time.

Vaccination is based on the knowledge that exposure to noninfectious antigens, derived from known pathogens, generates a mild primary immune response. The immune response to vaccination may not be perceived by the host as illness but still confers immune memory. When exposed to the corresponding pathogen to which an individual was vaccinated, the reaction is similar to a secondary exposure. Because each reinfection generates more memory cells and increased resistance to the pathogen, some vaccine courses involve one or more booster vaccinations to mimic repeat exposures.

The Lymphatic System

Lymph is the watery fluid that bathes tissues and organs and contains protective white blood cells but does not contain erythrocytes. Lymph moves about the body through the lymphatic system, which is made up of vessels, lymph ducts, lymph glands, and organs, such as tonsils, adenoids, thymus, and spleen.

Although the immune system is characterized by circulating cells throughout the body, the regulation, maturation, and intercommunication of immune factors occur at specific sites. The blood circulates immune cells, proteins, and other factors through the body. Approximately 0.1 percent of all cells in the blood are leukocytes, which include monocytes (the precursor of macrophages) and lymphocytes. Most cells in the blood are red blood cells. Cells of the immune system can travel between the distinct lymphatic and blood circulatory systems, which are separated by interstitial space, by a process called extravasation (passing through to surrounding tissue).

Recall that cells of the immune system originate from stem cells in the bone marrow. B cell maturation occurs in the bone marrow, whereas progenitor cells migrate from the bone marrow and develop and mature into naïve T cells in the organ called the thymus.

On maturation, T and B lymphocytes circulate to various destinations. Lymph nodes scattered throughout the body house large populations of T and B cells, dendritic cells, and macrophages (Figure 8.7.3.8). Lymph gathers antigens as it drains from tissues. These antigens then are filtered through lymph nodes before the lymph is returned to circulation. APCs in the lymph nodes capture and process antigens and inform nearby lymphocytes about potential pathogens.

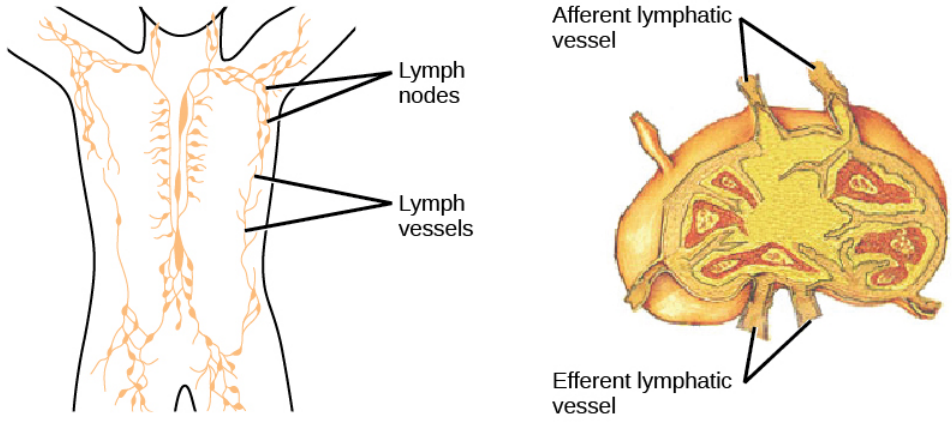


Figure 8.7.3.8: (a) Lymphatic vessels carry a clear fluid called lymph throughout the body. The liquid passes through (b) lymph nodes that filter the lymph that enters the node through afferent vessels and leaves through efferent vessels; lymph nodes are filled with lymphocytes that purge infecting cells. (credit a: modification of work by NIH; credit b: modification of work by NCI, NIH)

The spleen houses B and T cells, macrophages, dendritic cells, and NK cells (Figure 8.7.3.9). The spleen is the site where APCs that have trapped foreign particles in the blood can communicate with lymphocytes. Antibodies are synthesized and secreted by activated plasma cells in the spleen, and the spleen filters foreign substances and antibody-complexed pathogens from the blood. Functionally, the spleen is to the blood as lymph nodes are to the lymph.

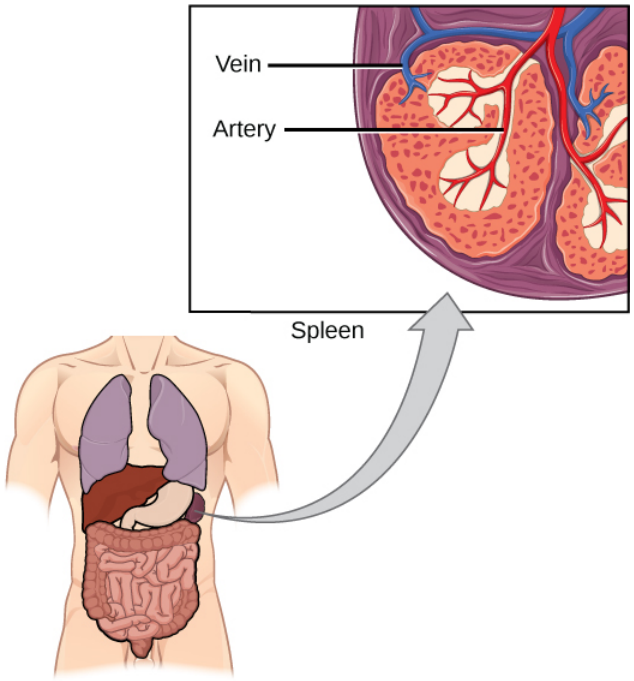


Figure 8.7.3.9: The spleen functions to immunologically filter the blood and allow for communication between cells corresponding to the innate and adaptive immune responses. (credit: modification of work by NCI, NIH)

Mucosal Immune System

The innate and adaptive immune responses compose the systemic immune system (affecting the whole body), which is distinct from the mucosal immune system. Mucosa associated lymphoid tissue (MALT) is a crucial component of a functional immune system because mucosal surfaces, such as the nasal passages, are the first tissues onto which inhaled or ingested pathogens are deposited. The mucosal tissue includes the mouth, pharynx, and esophagus, and the gastrointestinal, respiratory, and urogenital tracts.

Mucosal immunity is formed by MALT, which functions independently of the systemic immune system, and which has its own innate and adaptive components. MALT is a collection of lymphatic tissue that combines with epithelial tissue lining the mucosa throughout the body. This tissue functions as the immune barrier and response in areas of the body with direct contact to the external environment. The systemic and mucosal immune systems use many of the same cell types. Foreign particles that make their way to MALT are taken up by absorptive epithelial cells and delivered to APCs located directly below the mucosal tissue. APCs of the mucosal immune system are primarily dendritic cells, with B cells and macrophages having minor roles. Processed antigens displayed on APCs are detected by T cells in the MALT and at the tonsils, adenoids, appendix, or the mesenteric lymph nodes of the intestine. Activated T cells then migrate through the lymphatic system and into the circulatory system to mucosal sites of infection.

Immune Tolerance

The immune system has to be regulated to prevent wasteful, unnecessary responses to harmless substances, and more importantly, so that it does not attack "self." The acquired ability to prevent an unnecessary or harmful immune response to a detected foreign substance known not to cause disease, or self-antigens, is described as immune tolerance. The primary mechanism for developing immune tolerance to self-antigens occurs during the selection for weakly self-binding cells during T and B lymphocyte maturation. There are populations of T cells that suppress the immune response to self-antigens and that suppress the immune response after the infection has cleared to minimize host cell damage induced by inflammation and cell lysis. Immune tolerance is especially well developed in the mucosa of the upper digestive system because of the tremendous number of foreign substances (such as food proteins) that APCs of the oral cavity, pharynx, and gastrointestinal mucosa encounter. Immune tolerance is brought about by specialized APCs in the liver, lymph nodes, small intestine, and lung that present harmless antigens to a diverse population of regulatory T (T_{reg}) cells, specialized lymphocytes that suppress local inflammation and inhibit the secretion of stimulatory immune factors. The combined result of T_{reg} cells is to prevent immunologic activation and inflammation in undesired tissue compartments and to allow the immune system to focus on pathogens instead.

Section Summary

The adaptive immune response is a slower-acting, longer-lasting, and more specific response than the innate response. However, the adaptive response requires information from the innate immune system to function. APCs display antigens on MHC molecules to naïve T cells. T cells with cell-surface receptors that bind a specific antigen will bind to that APC. In response, the T cells differentiate and proliferate, becoming T_H cells or T_C cells. T_H cells stimulate B cells that have engulfed and presented pathogen-derived antigens. B cells differentiate into plasma cells that secrete antibodies, whereas T_C cells destroy infected or cancerous cells. Memory cells are produced by activated and proliferating B and T cells and persist after a primary exposure to a pathogen. If re-exposure occurs, memory cells differentiate into effector cells without input from the innate immune system. The mucosal immune system is largely independent of the systemic immune system but functions in parallel to protect the extensive mucosal surfaces of the body. Immune tolerance is brought about by T_{reg} cells to limit reactions to harmless antigens and the body's own molecules.

Art Connections

Figure **8.7.3.6**: The Rh antigen is found on Rh-positive red blood cells. An Rh-negative female can usually carry an Rh-positive fetus to term without difficulty. However, if she has a second Rh-positive fetus, her body may launch an immune attack that causes hemolytic disease of the newborn. Why do you think hemolytic disease is only a problem during the second or subsequent pregnancies?

Answer

If the blood of the mother and fetus mixes, memory cells that recognize the Rh antigen of the fetus can form in the mother late in the first pregnancy. During subsequent pregnancies, these memory cells launch an immune attack on the fetal blood cells of an Rh-positive fetus. Injection of anti-Rh antibody during the first pregnancy prevents the immune response from occurring.

Glossary

active immunity

an immunity that occurs as a result of the activity of the body's own cells rather than from antibodies acquired from an external source

adaptive immunity
a specific immune response that occurs after exposure to an antigen either from a pathogen or a vaccination

antibody
a protein that is produced by plasma cells after stimulation by an antigen; also known as an immunoglobulin

antigen
a macromolecule that reacts with cells of the immune system and which may or may not have a stimulatory effect

antigen-presenting cell (APC)
an immune cell that detects, engulfs, and informs the adaptive immune response about an infection by presenting the processed antigen on its cell surface

B cell
a lymphocyte that matures in the bone marrow

cell-mediated immune response
an adaptive immune response that is controlled by T cells

cytotoxic T lymphocyte (T_C)
an adaptive immune cell that directly kills infected cells via enzymes, and that releases cytokines to enhance the immune response

dendritic cell
an immune cell that processes antigen material and presents it on the surface of its cell in MHC class II molecules and induces an immune response in other cells

effector cell
a lymphocyte that has differentiated, such as a B cell, plasma cell, or cytotoxic T cell

helper T lymphocyte (T_H)
a cell of the adaptive immune system that binds APCs via MHC class II molecules and stimulates B cells or secretes cytokines to initiate the immune response

humoral immune response
the adaptive immune response that is controlled by activated B cells and antibodies

immune tolerance
an acquired ability to prevent an unnecessary or harmful immune response to a detected foreign body known not to cause disease

lymph
the watery fluid present in the lymphatic circulatory system that bathes tissues and organs with protective white blood cells and does not contain erythrocytes

memory cell
an antigen-specific B or T lymphocyte that does not differentiate into an effector cell during the primary immune response but that can immediately become an effector cell on reexposure to the same pathogen

major histocompatibility class (MHC) II molecule

a protein found on the surface of antigen-presenting cells that signals to immune cells whether the cell is normal or is infected or cancerous; it provides the appropriate template into which antigens can be loaded for recognition by lymphocytes

passive immunity

an immunity that does not result from the activity of the body's own immune cells but by transfer of antibodies from one individual to another

primary immune response

the response of the adaptive immune system to the first exposure to an antigen

secondary immune response

the response of the adaptive immune system to a second or later exposure to an antigen mediated by memory cells

T cell

a lymphocyte that matures in the thymus gland

Contributors and Attributions

- Samantha Fowler (Clayton State University), Rebecca Roush (Sandhills Community College), James Wise (Hampton University). Original content by OpenStax (CC BY 4.0; Access for free at https://cnx.org/contents/b3c1e1d2-83...4-e119a8aafbdd).

This page titled 8.7.3: Adaptive Immunity is shared under a CC BY license and was authored, remixed, and/or curated by .

- **17.3: Adaptive Immunity** by OpenStax is licensed CC BY 4.0.

8.7.4: Disruptions in the Immune System

A functioning immune system is essential for survival, but even the sophisticated cellular and molecular defenses of the mammalian immune response can be defeated by pathogens at virtually every step. In the competition between immune protection and pathogen evasion, pathogens have the advantage of more rapid evolution because of their shorter generation time, large population sizes and often higher mutation rates. Thus pathogens have evolved a diverse array of immune escape mechanisms. For instance, *Streptococcus pneumoniae* (the bacterium that causes pneumonia and meningitis) surrounds itself with a capsule that inhibits phagocytes from engulfing it and displaying antigens to the adaptive immune system. *Staphylococcus aureus* (the bacterium that can cause skin infections, abscesses, and meningitis) synthesizes a toxin called leukocidin that kills phagocytes after they engulf the bacterium. Other pathogens can also hinder the adaptive immune system. HIV infects T_H cells using their CD4 surface molecules, gradually depleting the number of T_H cells in the body (Figure 8.7.4.1); this inhibits the adaptive immune system's capacity to generate sufficient responses to infection or tumors. As a result, HIV-infected individuals often suffer from infections that would not cause illness in people with healthy immune systems but which can cause devastating illness to immune-compromised individuals.

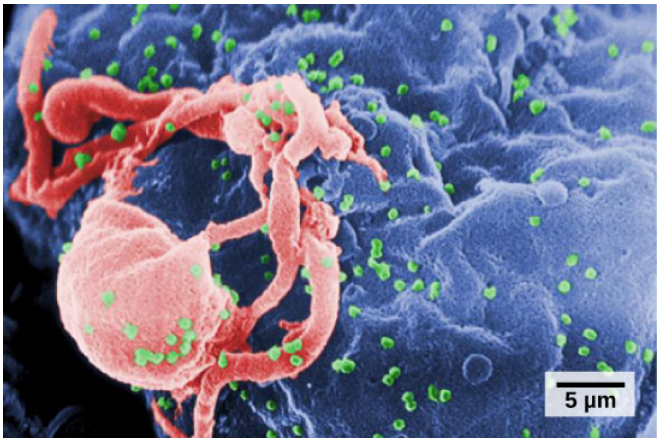


Figure 8.7.4.1: HIV (green) is shown budding from a lymphocyte cell (red) in culture. (credit: modification of work by C. Goldsmith, CDC; scale-bar data from Matt Russell)

Inappropriate responses of immune cells and molecules themselves can also disrupt the proper functioning of the entire system, leading to host-cell damage that can become fatal.

Immunodeficiency

Immunodeficiency is a failure, insufficiency, or delay in the response of the immune system, which may be acquired or inherited. Immunodeficiency can allow pathogens or tumor cells to gain a foothold and replicate or proliferate to high enough levels so that the immune system becomes overwhelmed. Immunodeficiency can be acquired as a result of infection with certain pathogens that attack the cells of the immune system itself (such as HIV), chemical exposure (including certain medical treatments such as chemotherapy), malnutrition, or extreme stress. For instance, radiation exposure can destroy populations of lymphocytes and elevate an individual's susceptibility to infections and cancer. Rarely, primary immunodeficiencies that are present from birth may also occur. For example, severe combined immunodeficiency disease (SCID) is a condition in which children are born without functioning B or T cells.

Hypersensitivities

A maladaptive immune response toward harmless foreign substances or self-antigens that occur after tissue sensitization is termed a hypersensitivity. Types of hypersensitivities include immediate, delayed, and autoimmune. A large proportion of the human population is affected by one or more types of hypersensitivity.

Allergies

The immune reaction that results from immediate hypersensitivities in which an antibody-mediated immune response occurs within minutes of exposure to a usually harmless antigen is called an allergy. In the United States, 20 percent of the population exhibits symptoms of allergy or asthma, whereas 55 percent test positive against one or more allergens. On initial exposure to a potential

allergen, an allergic individual synthesizes antibodies through the typical process of APCs presenting processed antigen to T_H cells that stimulate B cells to produce the antibodies. The antibody molecules interact with mast cells embedded in connective tissues. This process primes, or sensitizes, the tissue. On subsequent exposure to the same allergen, antibody molecules on mast cells bind the antigen and stimulate the mast cell to release histamine and other inflammatory chemicals; these chemical mediators then recruit eosinophils (a type of white blood cell), which also appear to be adapted to responding to parasitic worms (Figure 8.7.4.2). Eosinophils release factors that enhance the inflammatory response and the secretions of mast cells. The effects of an allergic reaction range from mild symptoms like sneezing and itchy, watery eyes to more severe or even life-threatening reactions involving intensely itchy welts or hives, airway constriction with severe respiratory distress, and plummeting blood pressure caused by dilating blood vessels and fluid loss from the circulatory system. This extreme reaction, typically in response to an allergen introduced to the circulatory system, is known as anaphylactic shock. Antihistamines are an insufficient counter to anaphylactic shock and if not treated with epinephrine to counter the blood pressure and breathing effects, this condition can be fatal.

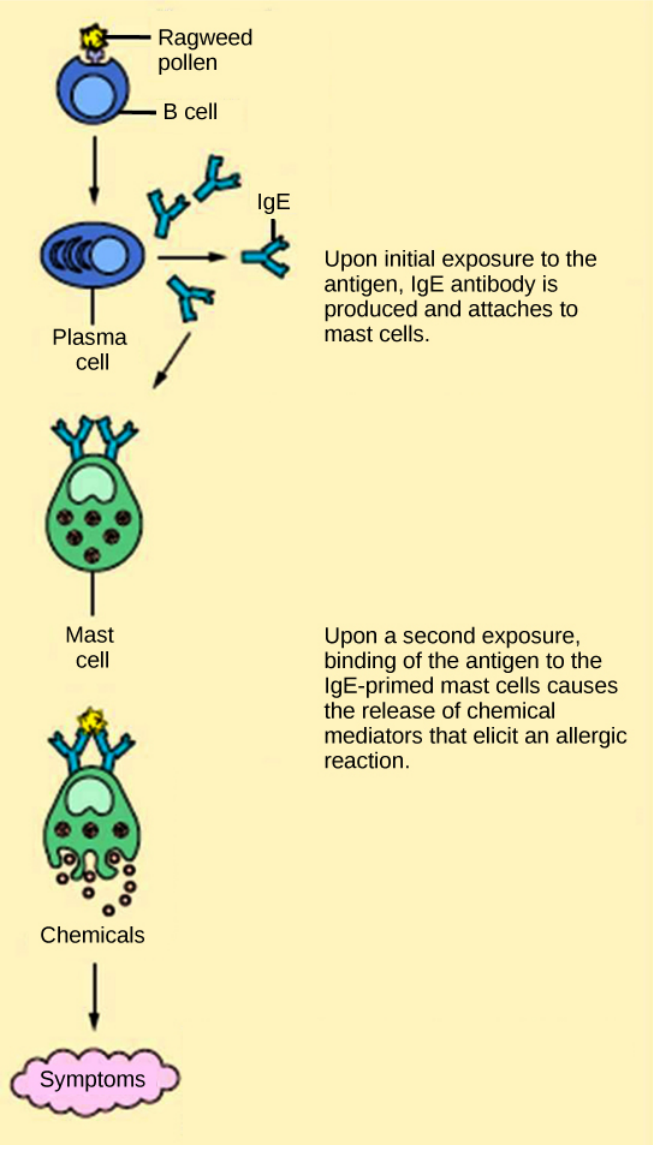


Figure 8.7.4.2: On first exposure to an allergen, an antibody is synthesized by plasma cells in response to a harmless antigen. The antibodies bind to mast cells, and on secondary exposure, the mast cells release histamines and other modulators that cause the symptoms of allergy. (credit: modification of work by NIH)

Delayed hypersensitivity is a cell-mediated immune response that takes approximately one to two days after secondary exposure for a maximal reaction. This type of hypersensitivity involves the T_H1 cytokine-mediated inflammatory response and may cause local tissue lesions or contact dermatitis (rash or skin irritation). Delayed hypersensitivity occurs in some individuals in response to contact with certain types of jewelry or cosmetics. Delayed hypersensitivity facilitates the immune response to poison ivy and is

also the reason why the skin test for tuberculosis results in a small region of inflammation on individuals who were previously exposed to *Mycobacterium tuberculosis*, the organism that causes tuberculosis.

CONCEPT IN ACTION

Try your hand at diagnosing an allergic reaction by selecting one of the interactive case studies at the World Allergy Organization website.

Autoimmunity

Autoimmunity is a type of hypersensitivity to self-antigens that affects approximately five percent of the population. Most types of autoimmunity involve the humoral immune response. An antibody that inappropriately marks self-components as foreign is termed an autoantibody. In patients with myasthenia gravis, an autoimmune disease, muscle-cell receptors that induce contraction in response to acetylcholine are targeted by antibodies. The result is muscle weakness that may include marked difficultly with fine or gross motor functions. In systemic lupus erythematosus, a diffuse autoantibody response to the individual's own DNA and proteins results in various systemic diseases (Figure 8.7.4.3). Systemic lupus erythematosus may affect the heart, joints, lungs, skin, kidneys, central nervous system, or other tissues, causing tissue damage through antibody binding, complement recruitment, lysis, and inflammation.

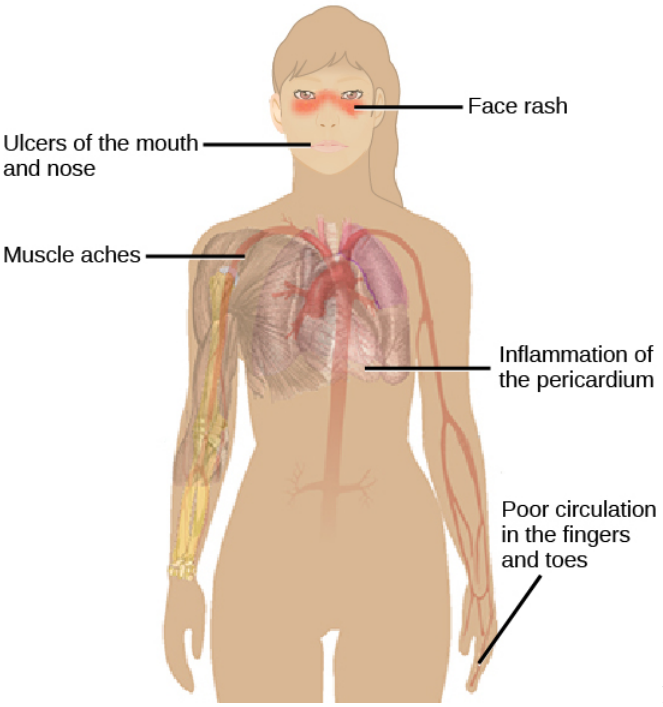


Figure 8.7.4.3: Systemic lupus erythematosus is characterized by autoimmunity to the individual's own DNA and/or proteins, which leads to varied dysfunction of the organs. (credit: modification of work by Mikael Häggström)

Autoimmunity can develop with time and its causes may be rooted in molecular mimicry, a situation in which one molecule is similar enough in shape to another molecule that it binds the same immune receptors. Antibodies and T-cell receptors may bind self-antigens that are structurally similar to pathogen antigens. As an example, infection with *Streptococcus pyogenes* (the bacterium that causes strep throat) may generate antibodies or T cells that react with heart muscle, which has a similar structure to the surface of *S. pyogenes*. These antibodies can damage heart muscle with autoimmune attacks, leading to rheumatic fever. Insulin-dependent (Type 1) diabetes mellitus arises from a destructive inflammatory T_H1 response against insulin-producing cells of the pancreas. Patients with this autoimmunity must be treated with regular insulin injections.

Summary

Immune disruptions may involve insufficient immune responses or inappropriate immune responses. Immunodeficiency increases an individual's susceptibility to infections and cancers. Hypersensitivities are misdirected responses either to harmless foreign

particles, as in the case of allergies, or to the individual's own tissues, as in the case of autoimmunity. Reactions to self-components may be the result of molecular mimicry.

Glossary

allergy
an immune reaction that results from immediate hypersensitivities in which an antibody-mediated immune response occurs within minutes of exposure to a harmless antigen

autoantibody
an antibody that incorrectly marks "self" components as foreign and stimulates the immune response

autoimmunity
a type of hypersensitivity to self-antigens

hypersensitivity
a spectrum of inappropriate immune responses toward harmless foreign particles or self-antigens; occurs after tissue sensitization and includes immediate-type (allergy), delayed-type, and autoimmunity

immunodeficiency
a failure, insufficiency, or delay at any level of the immune system, which may be acquired or inherited

Contributors and Attributions

- Samantha Fowler (Clayton State University), Rebecca Roush (Sandhills Community College), James Wise (Hampton University). Original content by OpenStax (CC BY 4.0; Access for free at https://cnx.org/contents/b3c1e1d2-83...4-e119a8aafbdd).

This page titled 8.7.4: Disruptions in the Immune System is shared under a CC BY license and was authored, remixed, and/or curated by .

- **17.4: Disruptions in the Immune System** by OpenStax is licensed CC BY 4.0.

8.7.E: The Immune System and Desease (Exercises)

17.1: Viruses

Viruses are acellular, parasitic entities that are not classified within any domain because they are not considered alive. They have no plasma membrane, internal organelles, or metabolic processes, and they do not divide. Instead, they infect a host cell and use the host's replication processes to produce progeny virus particles. Viruses infect all forms of organisms including bacteria, archaea, fungi, plants, and animals.

Review Questions

Which statement is true?

A. A virion contains DNA and RNA.
B. Viruses are acellular.
C. Viruses replicate outside of the cell.
D. Most viruses are easily visualized with a light microscope.

Answer

B

The viral _____ plays a role in attaching a virion to the host cell.

A. core
B. capsid
C. envelope
D. both b and c

Answer

D

Which statement is true of viral replication?

A. In the process of apoptosis, the cell survives.
B. During attachment, the virus attaches at specific sites on the cell surface.
C. The viral capsid helps the host cell produce more copies of the viral genome.
D. mRNA works outside of the host cell to produce enzymes and proteins.

Answer

B

Free Response

Why can't dogs catch the measles?

Answer

The virus cannot attach to dog cells because dog cells do not express the receptors for the virus or there is no cell within the dog that is permissive for viral replication.

Why is immunization after being bitten by a rabid animal so effective?

Answer

Rabies vaccine works after a bite because it takes two weeks for the virus to travel from the site of the bite to the central nervous system, where the most severe symptoms of the disease occur. The vaccine is able to cause an immune response in the body during this time that clears the infection before it reaches the nervous system.

17.2: Innate Immunity

Innate immunity is not caused by an infection or vaccination and depends initially on physical and chemical barriers that work on all pathogens, sometimes called the first line of defense. The second line of defense of the innate system includes chemical signals that produce inflammation and fever responses as well as mobilizing protective cells and other chemical defenses.

Review Questions

Which of the following is a barrier against pathogens provided by the skin?

A. low pH
B. mucus
C. tears
D. cilia

Answer

A

Although interferons have several effects, they are particularly useful against infections with which type of pathogen?

A. bacteria
B. viruses
C. fungi
D. helminths

Answer

B

Which innate immune system component uses MHC class I molecules directly in its defense strategy?

A. macrophages
B. neutrophils
C. NK cells
D. interferon

Answer

C

Free Response

Different MHC class I molecules between donor and recipient cells can lead to rejection of a transplanted organ or tissue. Suggest a reason for this.

Answer

If the MHC class I molecules expressed on donor cells differ from the MHC class I molecules expressed on recipient cells, NK cells may identify the donor cells as not normal and produce enzymes to induce the donor cells to undergo apoptosis, which would destroy the transplanted organ.

If a series of genetic mutations prevented some, but not all, of the complement proteins from binding antibodies or pathogens, would the entire complement system be compromised?

Answer

The entire complement system would probably be affected even when only a few members were mutated such that they could no longer bind. Because the complement involves the binding of activated proteins in a specific sequence, when one or more proteins in the sequence is absent, the subsequent proteins would be incapable of binding to elicit the complement's pathogen-destructive effects.

17.3: Adaptive Immunity

The adaptive immune response is a slower-acting, longer-lasting, and more specific response than the innate response. However, the adaptive response requires information from the innate immune system to function. APCs display antigens on MHC molecules to naïve T cells. T cells with cell-surface receptors that bind a specific antigen will bind to that APC. In response, the T cells differentiate and proliferate.

Review Questions

The humoral immune response depends on which cells?

A. T_C cells
B. B cells
C. B and T_H cells
D. T_C and T_H cells

Answer

C

The fact that the body does not normally mount an immune response to the molecules in food is an example of _____.

A. secondary immune response
B. immunological memory
C. immune tolerance
D. passive immunity

Answer

C

Foreign particles circulating in the blood are filtered by the _____.

A. spleen
B. lymph nodes
C. MALT
D. lymph

Answer

A

Free Response

How do B and T cells differ with respect to antigens that they bind?

Answer

T cells bind antigens that have been digested and embedded in MHC molecules by APCs. In contrast, B cells function as APCs to bind intact, unprocessed antigens.

Why is the immune response after reinfection much faster than the adaptive immune response after the initial infection?

Answer

Upon reinfection, the memory cells will immediately differentiate into plasma cells and CTLs without input from APCs or T_H cells. In contrast, the adaptive immune response to the initial infection requires time for naïve B and T cells with the appropriate antigen specificities to be identified and activated.

17.4: Disruptions in the Immune System

A functioning immune system is essential for survival, but even the sophisticated cellular and molecular defenses of the mammalian immune response can be defeated by pathogens at virtually every step. In the competition between immune protection and pathogen evasion, pathogens have the advantage of more rapid evolution because of their shorter generation time, large population sizes and often higher mutation rates. Thus pathogens have evolved a diverse array of immune escape mechanisms.

Review Questions

Allergy to pollen is classified as _____.

A. an autoimmune reaction
B. immunodeficiency
C. delayed hypersensitivity
D. immediate hypersensitivity

Answer

D

A potential cause of acquired autoimmunity is _____.

A. tissue hypersensitivity
B. molecular mimicry
C. histamine release
D. radiation exposure

Answer

B

Autoantibodies are probably involved in _____.

A. reactions to poison ivy
B. pollen allergies
C. systemic lupus erythematosus
D. HIV/AIDS

Answer

C

Free Response

Some photographers develop a sensitivity to certain film developing chemicals leading to severe rashes on their hands such that they are unable to work with them. Explain what is probably happening.

Answer

This is probably a delayed sensitivity reaction to one or more chemicals in the developer. An initial exposure would have sensitized the individual to the chemical and then subsequent exposures will induce a delayed inflammation reaction a day or two after exposure.

This page titled 8.7.E: The Immune System and Desease (Exercises) is shared under a CC BY license and was authored, remixed, and/or curated by .

- **17.E: The Immune System and Desease (Exercises)** by OpenStax is licensed CC BY 4.0.

SECTION OVERVIEW

8.8: Biotechnology

Topic hierarchy

Thumbnail: A group of Genetically modified GloFish fluorescent fish. (www.glofish.com).

This page titled 8.8: Biotechnology is shared under a CC BY license and was authored, remixed, and/or curated by .

CHAPTER OVERVIEW

9: Basic Techniques

The environment of a cell is very complex, making it difficult to study individual reactions, enzymes, or pathways in situ. The traditional approach used by biochemists for the study of these things is to isolate molecules, enzymes, DNAs, RNAs, and other items of interest so they can be analyzed independently of the millions of other processes occurring simultaneously. Today, these approaches are used side by side with newer methods that allow us to understand events inside cells on a larger scale- for example, determining all the genes that are being expressed at a given time in specific cells. In this section we take a brief look at some commonly used methods used to study biological molecules and their interactions.

9.1: Cell Lysis
9.2: Fractionation and Chromatography Techniques
9.3: Electrophoresis
9.4: Detection, identification and quantitation of specific nucleic acids and proteins
9.5: Transcriptomics
9.6: Isolating Genes
9.7: Polymerase Chain Reaction (PCR)
9.8: Reverse Transcription
9.9: Genome Editing (CRISPR)
9.10: Membrane Dynamics (FRAP)

Thumbnail: A western blot. Image used with permission (CC BY-SA 3.0; Magnus Manske).

This page titled 9: Basic Techniques is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

9.1: Cell Lysis

Source: BiochemFFA_8_1.pdf. The entire textbook is available for free from the authors at http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

To separate compounds from cellular environments, one must first break open (lyse) the cells. Cells are broken open, in buffered solutions, to obtain a lysate. There are several ways of accomplishing this.

- **Osmotic shock and enzymes:** One way to lyse cells is by lowering the ionic strength of the medium the cells are in. This can cause cells to swell and burst. Mild surfactants may be used to disrupt membranes. Most bacteria, yeast, and plant tissues are resistant to osmotic shocks, because of the presence of cell walls, and stronger disruption techniques are usually required. Enzymes may be useful in helping to degrade the cell walls. Lysozyme, for example, is very useful for breaking down bacterial walls. Other enzymes commonly employed include cellulase (plants), proteases, mannases, and others.
- **Mechanical disruption:** Mechanical agitation may be employed in the form of beads that are shaken with a mixture of cells. In this method, cells are bombarded with tiny, glass beads that break the cells open. Sonication (20-50 kHz sound waves) provides an alternative type of agitation that can be effective. The method is noisy, however, and generates heat that can be problematic for heat-sensitive compounds.
- **Pressure disruption:** Another means of disrupting cells involves using a “cell bomb”. In this method, cells are placed under very high pressure (up to 25,000 psi) and then the pressure is rapidly released. The rapid pressure change causes dissolved gases in cells to be released as bubbles which, in turn, break open cells.
- **Cryopulverization:** Cryopulverization is often employed for samples having a tough extracellular matrix, such as connective tissue, seed, and cartilage. In this technique, tissues are frozen using liquid nitrogen and then impact pulverization (typically, grinding, using a mortar and pestle or a powerful electric grinder) is performed. The powder so obtained is then suspended in the appropriate buffer.



Figure 8.1 - A sonicator in use. Wikipedia

Whatever method is employed to create a lysate, crude fractions obtained from it must be further processed via fractionation.

This page titled 9.1: Cell Lysis is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **8.1: Cell Lysis** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

9.2: Fractionation and Chromatography Techniques

Fractionation of samples, as the name suggests, is a process of separating out the components or fractions of the lysate. Fractionation typically begins with centrifugation of the lysate. Using low-speed centrifugation, one can remove cell debris, leaving a supernatant containing the contents of the cell. By using successively higher centrifugation speeds (and resulting g forces) it is possible to separate out different cellular components, like nuclei, mitochondria, etc., from the cytoplasm. These may then be separately lysed to release molecules that are specific to the particular cellular compartment. The soluble fraction of any lysate can, then, be further separated into its constituents using various methods.


Figure **9.2.1**: A high-speed centrifuge can be used to obtain different cell fractions from a crude lysate. Wikipedia

Column Chromatography

One powerful method used for this purpose is chromatography. We will consider several chromatographic approaches. Chromatography is used to separate out the components of a mixture based on differences in their size, charge or other characteristics. During chromatography, the mobile phase (buffer or other solvent) moves through the stationary phase (usually a solid matrix) carrying the components of the mixture. Separation of the components is achieved, because the different components move at different rates, for reasons that vary, depending on the type of chromatography used. We will consider several different kinds of chromatography to illustrate this process.

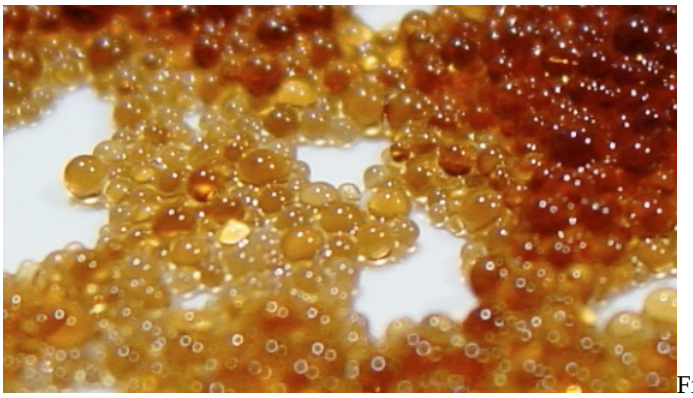
- Ion exchange chromatography
- Gel exclusion chromatography
- Affinity chromatography
- HPLC


Figure **9.2.2**: An ion exchange column apparatus.Wikipedia

These variations on chromatography are performed with the stationary phase held within so-called columns (Figure **9.2.1**). These are tubes containing the stationary phase (also called the “support” or solid phase).

Supports are composed of tiny beads suspended in buffer (Figure **9.2.3**) and are designed to exploit the chemistry or size differences of the components of the samples and thus provide a means of separation. Columns are “packed” or filled with the support, and a

buffer or solvent carries the mixture of compounds to be separated through the support. Molecules in the sample interact differentially with the support and, consequently, travel through it at different speeds, thus enabling separation.


Figure 9.2.3: Ion exchange beads.Wikipedia

Ion exchange chromatography

In ion exchange chromatography, the support consists of tiny beads to which are attached chemicals possessing a charge. Before use, the beads are equilibrated in a solution containing an appropriate counter-ion to the charged molecule on the bead. Figure 9.2.5 shows the repeating unit of polystyrolsulfonate, a compound used as a cation exchange resin. As you can see, this molecule is negatively charged, and thus the beads would be equilibrated in a buffer containing a positively charged ion, say sodium. In the suspension, the negatively charged polystyrolsulfonate is unable to leave the beads, due to its covalent attachment, but the counter-ions (sodium) can be "exchanged" for molecules of the same charge.

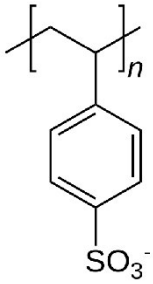


Figure 9.2.4: Polystyrolsulfonate, a cation exchange resin. Wikipedia

Exchanges

Thus, a cation exchange column will have positively charged counter-ions and negatively charged molecules covalently attached to the beads. Positively charged compounds from a cell lysate passed through the column will exchange with the counter-ions and "stick" to the negatively charged compounds covalently attached to the beads. Molecules in the sample that are neutral in charge or negatively charged will pass quickly through the column. At this point, only positively charged molecules from the original sample would be bound to the column. These may then be washed off, or eluted, by using buffers containing high concentrations of salt. Under these conditions, the interaction between the positively charged molecules and the polystyrosulfonate would be disrupted, allowing the molecules that were bound to the column to be recovered.

Anion exchange

On the other hand, in anion exchange chromatography, the chemicals attached to the beads are positively charged and the counterions are negatively charged (chloride, for example). Negatively charged molecules in the cell lysate will "stick" and other molecules will pass through quickly. To remove the molecules "stuck" to a column, one simply needs to add a high concentration of counter-ions to release them.

Uses

Ion exchange resins are useful for separating charged from uncharged, or oppositely charged, biomolecules in solution. The resins have a variety of other applications, including water purification and softening. Figure 9.2.5 shows use of a polystyrolsulfonate polymer in removing calcium for water softening.

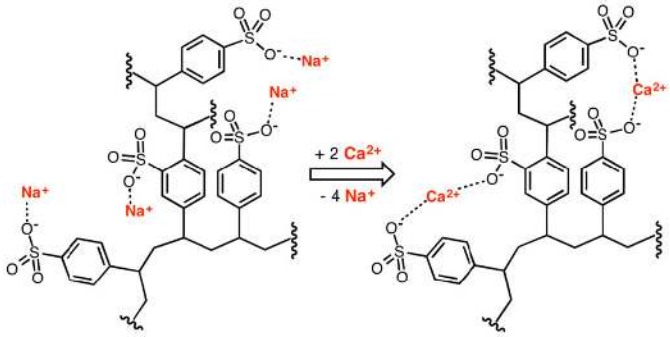


***Figure* 9.2.5**: *Removal of calcium ions by an ion exchanger. Wikipedia*

Size Exclusion Chromatography

Size exclusion chromatography (also called molecular exclusion chromatography, gel exclusion chromatography, or gel filtration chromatography) is a low resolution separation method that employs beads with tiny "tunnels" in them that each have a precise opening. The size of the opening is referred to as an "exclusion limit," which means that molecules above a certain molecular weight will not be able to pass through the tunnels. Molecules with physical sizes larger than the exclusion limit do not enter the tunnels and pass through the column relatively quickly, in the spaces outside the beads. Smaller molecules, which can enter the tunnels, do so, and thus, have a longer path that they take in passing through the column and elute last (Figure **9.2.6**).

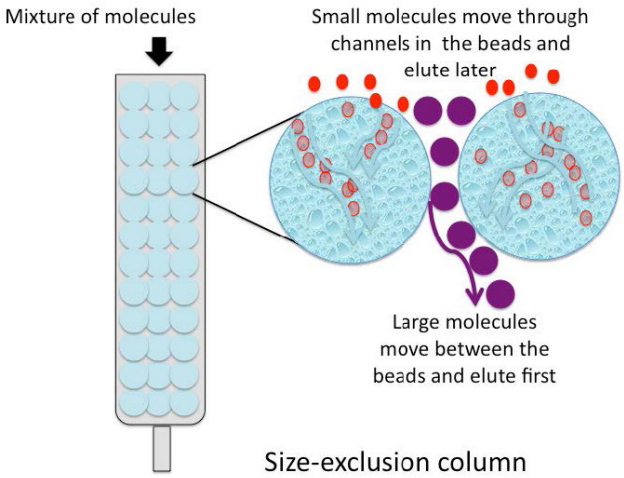


Figure **9.2.6**: Separation of molecules by size in a size-exclusion (aka gel filtration) column

Figure **9.2.7** shows a profile of a group of proteins separated by size exclusion chromatography using beads with an exclusion limit of about 30,000 Daltons. Proteins 30,000 in molecular weight or larger elute in the void volume (left) while smaller proteins elute later (middle and right).

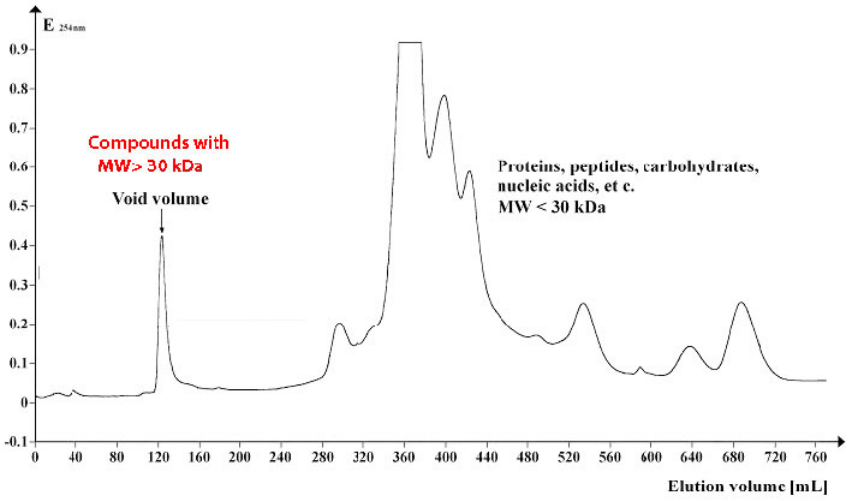


Figure 9.2.7: Result of a size exclusion separation. Wikipedia

Affinity chromatography

Affinity chromatography is a very powerful and selective technique that exploits the binding affinities of sample molecules (typically proteins) for molecules covalently linked to the support beads. In contrast to ion-exchange chromatography, where all molecules of a given charge would bind to the column, affinity chromatography exploits the specific binding of a protein or proteins to a ligand that is immobilized on the beads in the column.

For example, if one wanted to separate all of the proteins in a cell lysate that bind to ATP from proteins that do not bind ATP, one could use a column that has ATP attached to the support beads and pass the sample through the column. All proteins that bind ATP will "stick" to the column, whereas those that do not bind ATP will pass quickly through it. The bound proteins may then be released from the column by adding a solution of ATP that will displace the bound proteins by competing, for the proteins, with the ATP attached to the column matrix.

Histidine tagging

Histidine tagging (His-tagging) is a special kind of affinity chromatography and is a powerful tool for isolating a recombinant protein from a cell lysate. His-tagging relies on altering the DNA coding region for a protein to add a series of at least six histidine residues to the amino or carboxyl terminal of the encoded protein. This "His-Tag" is useful in purifying the tagged protein because histidine side chains can bind to nickel or cobalt ions. Separation of His-tagged proteins from a cell lysate is relatively easy (Figure 9.2.8).Passing the crude cell lysate through a column with nickel or cobalt attached to beads allows the His-tagged proteins to "stick," while the remaining cell proteins all pass quickly through. The His-tagged proteins are then eluted by addition of imidazole to the column. Imidazole, which resembles the side chain of histidine, competes with the His-tagged proteins and displaces them from the column. Although non-tagged proteins in the lysate may also contain histidine as part of their sequence, they will not bind to the column as strongly as the His-tagged protein and will, thus, be displaced at lower imidazole concentrations than needed to elute the His-tagged protein. Surprisingly, many His-tagged proteins appear to function normally despite the added histidines, but if needed, the histidine tags may be cleaved from the purified protein by treatment with a protease that excises the added histidines, allowing the recovery of the desired protein with its native sequence.

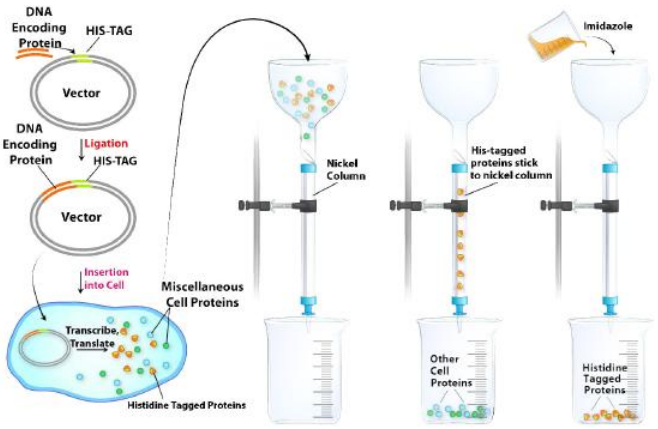


Figure **9.2.8**: *Affinity chromatographic purification of a protein by histidine tagging.Image by Aleia Kim*

HPLC

High performance liquid chromatography (HPLC) is a powerful tool for separating a variety of molecules based on their differential polarities (Figure **9.2.9**). A more efficient form of column chromatography, it employs columns with tightly packed supports and very tiny beads such that flow of solvents/buffers through the columns requires high pressures. The supports used may be polar (normal phase separation) or non-polar (reverse phase separation). In normal phase separations, non-polar molecules elute first followed by the more polar compounds. This order is switched in reverse phase chromatography. Of the two, reverse phase is much more commonly employed due to more reproducible chromatographic profiles (separations) that it typically produces.



Figure **9.2.9**: *HPLC: Pumps on left/Column in center/Detector on the right. Wikipedia*

This page titled 9.2: Fractionation and Chromatography Techniques is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

9.3: Electrophoresis

Electrophoresis uses an electric field applied across a gel matrix to separate large molecules such as DNA, RNA, and proteins by charge and size. Samples are loaded into the wells of a gel matrix that can separate molecules by size and an electrical field is applied across the gel. This field causes negatively charged molecules to move towards the positive electrode. The gel matrix, itself, acts as a sieve, through which the smallest molecules pass rapidly, while longer molecules are slower-moving.

For DNA and RNA, sorting molecules by size in this way is trivial, because of the uniform negative charge on the phosphate backbone. For proteins, which vary in their charges, a clever trick must be employed to make them mimic nucleic acids - see polyacrylamide gel electrophoresis (PAGE) below. Different kinds of gels have different pore sizes. Like sieves with finer or coarser meshes, some gels do a better job of separating smaller molecules while others work better for larger ones. Gel electrophoresis may be used as a preparative technique (that is, when purifying proteins or nucleic acids), but most often it is used as an analytical tool.

Agarose Gel Electrophoresis

Agarose gel electrophoresis is a technique used to separate nucleic acids primarily by size. Agarose is a polysaccharide obtained from seaweeds (Figure 8.11). It can be dissolved in boiling buffer and poured into a tray, where it sets up as it cools (Figure 8.12) to form a slab. Agarose gels are poured with a comb in place to make wells into which DNA or RNA samples are placed after the gel has solidified. The gel is immersed in a buffer and a current is applied across the slab. Double-stranded DNA has a uniform negative charge that is independent of the sequence composition of the molecule. Therefore, if DNA fragments are placed in an electric field they will migrate from the cathode (-) towards the anode (+). The rate of migration is directly dependent on the ability of each DNA molecule to worm or wiggle its way through the sieving gel. The agarose matrix provides openings for macromolecules to move through. The largest macromolecules have the most difficult time navigating through the gel, whereas the smallest macromolecules slip through it the fastest.

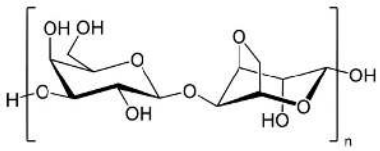
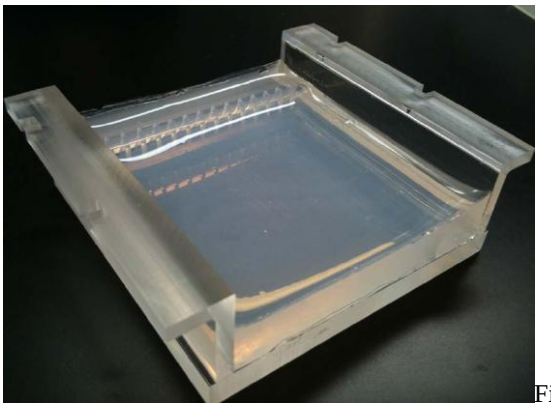


Figure 8.11 - Structure of the agarose polysaccharide. Wikipedia

Because electrophoresis uses an electric current as a force to drive the molecules through the matrix, the molecules being separated must be charged. Since the size to charge ratio for DNA and RNA is constant for all sizes of these nucleic acids, the molecules simply sort on the basis of their size - the smallest move fastest and the largest move slowest.

All fragments of a given size will migrate the same distance on the gel, forming the so-called “bands” on the gel. Visualization of the DNA fragments in the gel is made possible by addition of a dye, such as ethidium bromide, which intercalates between the bases and fluoresces when viewed under ultraviolet light (Figure 8.13) By running reference DNAs of known sizes alongside the samples, it is possible to determine the sizes of the DNA fragments in the sample. It is useful to note that, by convention, DNA fragments are not described by their molecular weights (unlike proteins), but by their length in base-pairs(bp) or kilobases (kb).

 Figure 8.12 - Agarose gel electrophoresis separation of DNA - orange bands are DNA fragments. Wikipedia

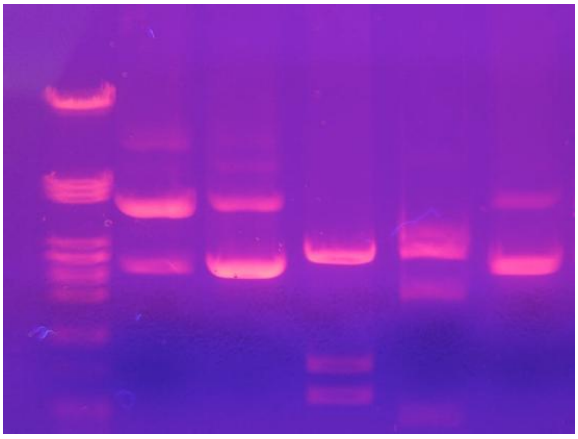


Figure 8.13 - DNA bands visualized with ethidium bromide staining. Wikipedia

Polyacrylamide gel electrophoresis (PAGE)

Like DNA and RNA, proteins are large macromolecules, but unlike nucleic acids, proteins are not necessarily negatively charged. The charge on each protein depends on its unique amino acid sequence. Thus, the proteins in a mixture will not necessarily all move towards the anode.

Additionally, whereas double-stranded DNA is rod-shaped, most proteins are globular (folded). Further, proteins are considerably smaller than nucleic acids, so the openings of the matrix of the agarose gel are simply too large to effectively provide separation. Consequently, unaltered (native) proteins are not very good prospects for electrophoresis on agarose gels. To separate proteins by mass using electrophoresis, one must make several modifications.

Gel matrix

First, a matrix made by polymerizing and cross-linking acrylamide units is employed. A monomeric acrylamide (Figure 8.14) is polymerized and the polymers are cross-linked using N,N'-Methylene-bisacrylamide (Figure 8.15) to create a mesh-like structure. One can adjust the size of the openings of the matrix/mesh readily by changing the percentage of acrylamide in the reaction. Higher percentages of acrylamide give smaller openings and are more effective for separating smaller molecules, whereas lower percentages of acrylamide are used when resolving mixtures of larger molecules. (Note: polyacrylamide gels are also used to separate small nucleic acid fragments, with some acrylamide gels capable of separating pieces of DNA that differ in length by just one nucleotide.)

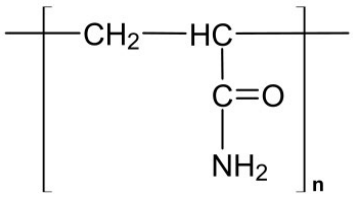


Figure 8.14 - Acrylamide monomer. Wikipedia

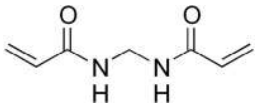


Figure 8.15 - N,N'-Methylenebisacrylamide - acrylamide crosslinking reagent. Wikipedia

Charge alteration by SDS

A second consideration is that proteins must be physically altered to "present" themselves to the matrix like the negatively charged rods of DNA. This is accomplished by treating the proteins with the anionic detergent, SDS (sodium dodecyl sulfate). SDS denatures the proteins so they assume a rod-like shape and the SDS molecules coat the proteins such that the exterior surface is loaded with negative charges, masking the original charges on the proteins and making the charge on the proteins more proportional to their mass, like the backbone of DNA.

Since proteins typically have disulfide bonds that prevent them from completely unfolding in detergent, samples are boiled with mercaptoethanol to break the disulfide bonds and ensure the proteins are as rod-like as possible in the SDS. Reagents like mercaptoethanol (and also dithiothreitol) are sulfhydryl-containing reagents that become oxidized as they reduce disulfide bonds in other molecules (see Figure 8.16)

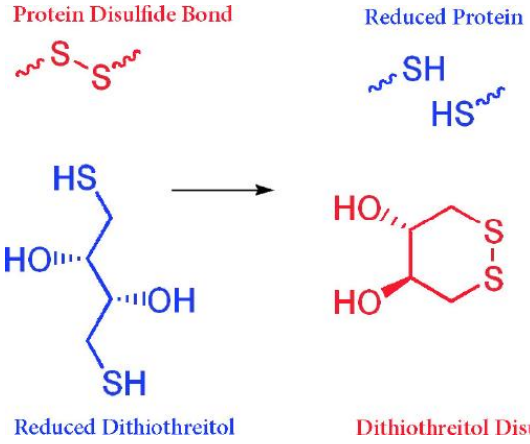


Figure 8.16 - Reduction of disulfide bonds by dithiothreitol. Wikipedia

Stacking Gel

A third consideration is that a "stacking gel" may be employed at the top of a polyacrylamide gel to provide a way of compressing the samples into a tight band before they enter the main polyacrylamide gel (called the resolving gel). Just like DNA fragments in agarose gel electrophoresis get sorted on the basis of size (largest move slowest and smallest move fastest), the proteins migrate through the gel matrix at velocities inversely related to their size. Upon completion of the electrophoresis, proteins may be visualized by staining with compounds that bind to proteins, like Coomassie Brilliant Blue (Figure 8.17) or silver nitrate.

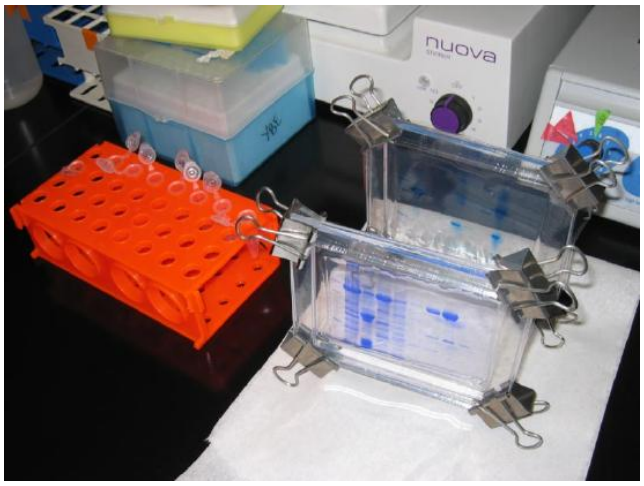


Figure 8.17 - Two SDS-PAGE gels - Proteins are the blue bands (stained with Coomassie Blue). Wikipedia

Non-denaturing gel electrophoresis

The SDS_PAGE technique described above is the commonest method used for electrophoretic separation of proteins. In some situations, however, proteins may be resolved on so-called “native” gels, in the absence of SDS. Under these conditions, the movement of proteins through the gel will be affected not simply by their mass, but by their charge at the pH of the gel, as well. Proteins complexed with other molecules may move as single entity, allowing the isolation of the binding partners of proteins of interest.

Isoelectric focusing

Proteins vary considerably in their charges and, consequently, in their pI values (pH at which their charge is zero). This can be exploited to separate proteins in a mixture. Separating proteins by isoelectric focusing requires establishment of a pH gradient in a tube containing an acrylamide gel matrix. The pore size of the gel is adjusted to be large, to reduce the effect of sieving based on size. Molecules to be separated are applied to the gel containing the pH gradient and an electric field is applied. Under these conditions, proteins will move according to their charge.

Positively charged molecules, for example, move towards the negative electrode, but since they are traveling through a pH gradient, as they pass through it, they reach a region where their charge is zero and, at that point, they stop moving. They are at that point attracted to neither the positive nor the negative electrode and are thus “focused” at their pI (Figure 8.18). Using isoelectric focusing, it is possible to separate proteins whose pI values differ by as little as 0.01 units.

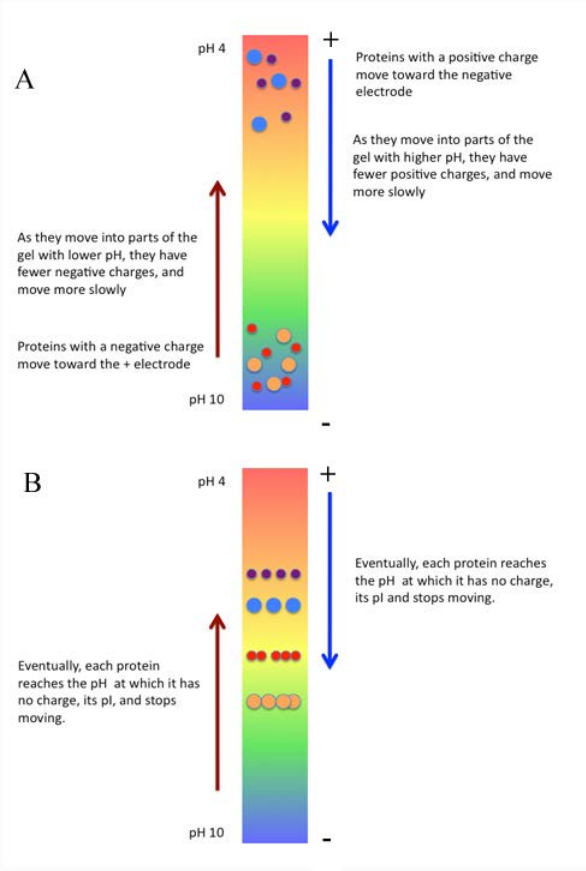


Figure 8.18 - Isoelectric focusing: A. At the start of the run; B. at the end of the run

2D gel electrophoresis

Both SDS-PAGE and isoelectric focusing are powerful techniques, but a clever combination of the two is a powerful tool of proteomics - the science of studying all of the proteins of a cell/tissue simultaneously. In 2-D gel electrophoresis, a lysate is first prepared from the cells of interest. The proteins in the lysate are separated first by their pI, through isoelectric focusing and then by size by SDS-PAGE.

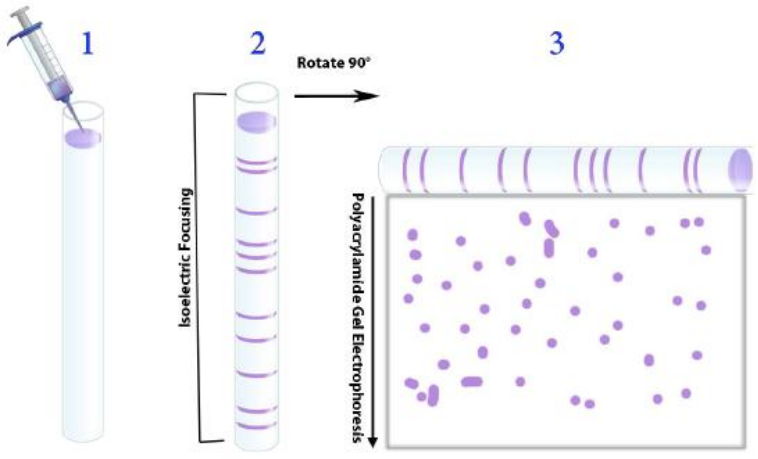


Figure 8.19 - Scheme for performing 2-D gel analysis. Image by Aleia Kim

The mixture of proteins is first applied to a tube or strip (Figure 8.19, Step 1) where isoelectric focusing is performed to separate the proteins by their pI values (Step 2). Next, as shown in the figure, the gel containing the proteins separated by their pIs is turned on its side and applied along the top of a polyacrylamide slab for SDS-PAGE to separate on the basis of size (Step 3). The proteins

in the isoelectric focusing matrix are electrophoresed into the polyacrylamide gel and separated on the basis of size. The product of this analysis is a 2-D gel as shown in Figure 8.20.The power of 2-D gel electrophoresis is that virtually every protein in a cell can be separated and appear on the gel as a spot defined by its unique size and pI. In the figure, spots in the upper left correspond to large positively charged proteins, whereas those in the lower right are small negatively charged ones. Every spot on a 2-D gel can be eluted and identified by using high throughput mass spectrometry. This is particularly powerful when one compares protein profiles between different tissues or between control and treated samples of the same tissue.

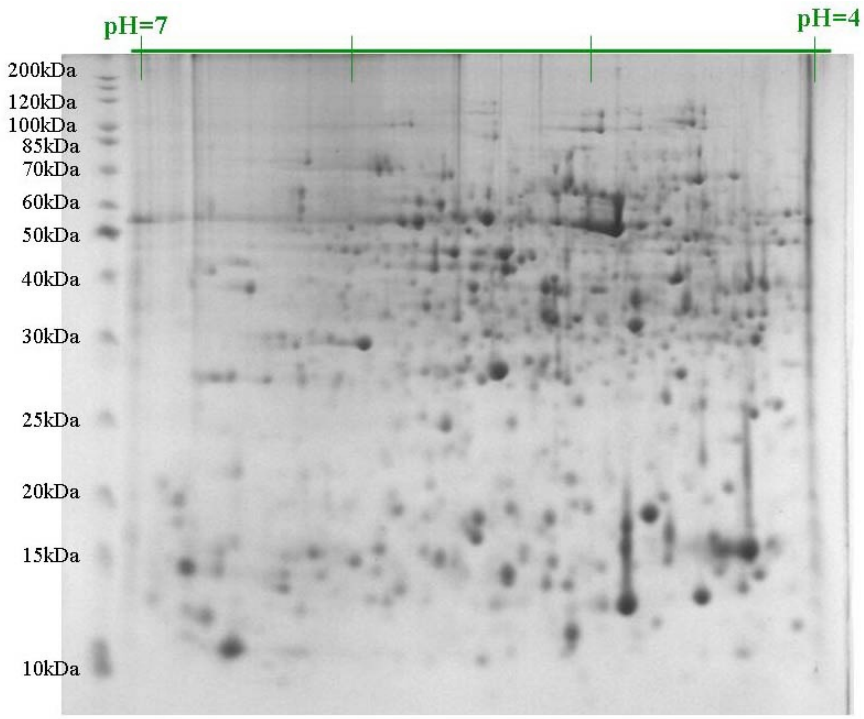


Figure 8.20 - Result of 2-D gel electrophoresis separation. Wikipedia

Protein profiles comparison

Comparison of 2-D gels of proteins from non-cancerous tissue and proteins from a cancerous tissue of the same type provides a quick identification of proteins whose level of expression differs between the two. Information such as this can be useful in designing treatments or in understanding the mechanism(s) by which the cancer develops.

This page titled 9.3: Electrophoresis is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **8.3: Electrophoresis** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

9.4: Detection, identification and quantitation of specific nucleic acids and proteins

While gel electrophoresis can be used to resolve molecules in a mixture, by itself, the technique does not permit the detection and identification of specific nucleic acid sequences or proteins. For example, the 2-D gel shown above clearly separates a large number of proteins in a sample into individual spots. However, if we wanted to know whether a specific protein was present, we could not tell by simply looking at the gel. Likewise, in an agarose gel, while bands of DNA could be assigned a size, one could not distinguish between two DNAs of different sequence if they were both the same length in base-pairs. One way to detect the presence of a particular nucleic acid or protein is dependent on transferring the separated molecules from the gels onto a membrane made of nitrocellulose or nylon to create a "blot" and probing for the molecule(s) of interest using reagents that specifically bind to those molecules. The next section will discuss how this can be done for nucleic acids as well as for proteins.

Southern and Northern Blots

The Southern blot is named for its inventor, Oxford professor, Edwin Southern, who came up with a protocol for transferring DNA fragments from a gel onto a nitrocellulose sheet and detecting a specific DNA sequence among the bands on the blot. As shown in Figure 8.21, the method works as follows. A mixture of DNA molecules (often DNA that has been cut into smaller fragments using restriction endonucleases) is loaded on an agarose gel, as usual. After the gel run is complete, the DNA bands are transferred from the gel onto a membrane. This can be achieved by capillary transfer, where the gel is placed in contact with a piece of membrane and buffer is pulled through the gel by wicking it up into a stack of absorbent paper placed above the membrane. As the buffer moves, it carries with it the DNA fragments. The DNA binds to the membrane leaving a "print" of DNA fragments that exactly mirrors their positions in the gel. The blotting membrane may be treated with UV light, heat, or chemicals to firmly attach the DNA to the membrane.

Next, a probe, or visualizing agent specific for the molecule of interest is added to the membrane. In Figure 8.21, this is called a labeled probe. The probes in a Southern blot are pieces of DNA designed to be complementary to the desired target sequence. If the sequence of interest is present on the blot, the probe, which is complementary to it, can base-pair (hybridize) with it. The blot is then washed to remove all unbound probe. Probes are labeled with radioactivity or with other chemical reagents that allow them to be easily detected when bound to the blot, so it is possible to visually determine whether the probe has bound to any of the DNA bands on the blot. Given that the Southern blot relies on specific base-pairing between the probe and the target sequence, it is easy to adapt the technique to detect specific RNA molecules, as well. The modification of this method to detect RNAs was jokingly named a "northern" blot.

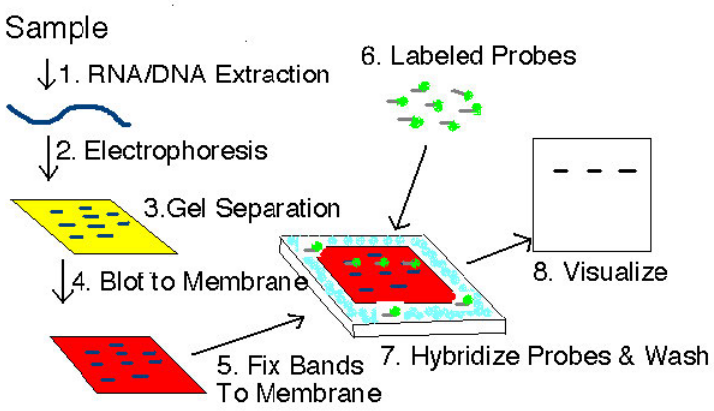


Figure 8.21 - Northern or Southern blot scheme. Southern blotting adds strand denaturation between steps 4 and 5. Wikipedia

Western Blots

Proteins cannot, for obvious reasons, be detected through base-pairing with a DNA probe, but protein blots, made by transferring proteins, separated on a gel, onto a membrane, can be probed using specific antibodies against a particular protein of interest. Protein detection usually employs two antibodies, the first of which is not labeled. The label is on the second antibody, which is

designed to recognize only the first antibody in a piggyback fashion. The first antibody specifically binds to the protein of interest on the blot and the second antibody recognizes and binds the first antibody. The second antibody commonly carries an enzyme or reagent which can cause a reaction to produce a color upon further treatment. In the end, if the molecule of interest is in the original mixture, it will “light” up and reveal itself on the blot. This variation on the blotting theme was dubbed a western blot (Figure 8.22).

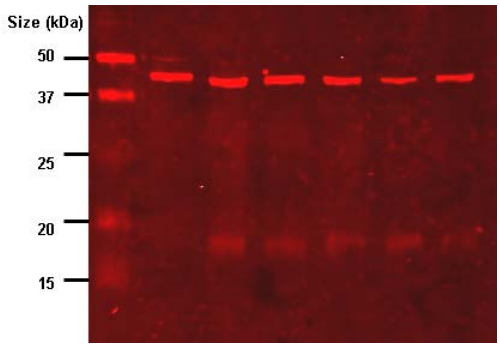


Figure 8.22 - Result of a western blot analysis. Wikipedia

In each of the blots described above, binding of the probe to the target molecule allows one to determine whether the target sequence or protein was in the sample. Although blots are designed to be used for detection, rather than for precise quantitation, it is possible to obtain estimates of the abundance of the target molecule from densitometry measurements of signal intensity.

Microarrays

2-D gels are a way of surveying a broad spectrum of protein molecules simultaneously. One approach to doing something similar with DNA or RNA involves what are called microarrays. Microarrays are especially useful for monitoring the expressions of thousands of genes, simultaneously. Where a northern blot would allow the identification of a single mRNA from a mixture of mRNAs, a microarray experiment can allow the simultaneous identification of thousands of mRNAs that may be made by a cell at a given time. It is also possible to perform quantitation much more reliably than with a blot.

Microarrays employ a glass slide, or chip, to which are attached short sequences of single-stranded DNA, arranged in a grid, or matrix (Figure 8.23) Each position in the grid corresponds to a unique gene. That is, the DNA sequence at this spot is part of the sequence of a specific gene. Each spot on the grid has multiple identical copies of the same sequence. The gene sequence immobilized at each position in the grid is recorded.

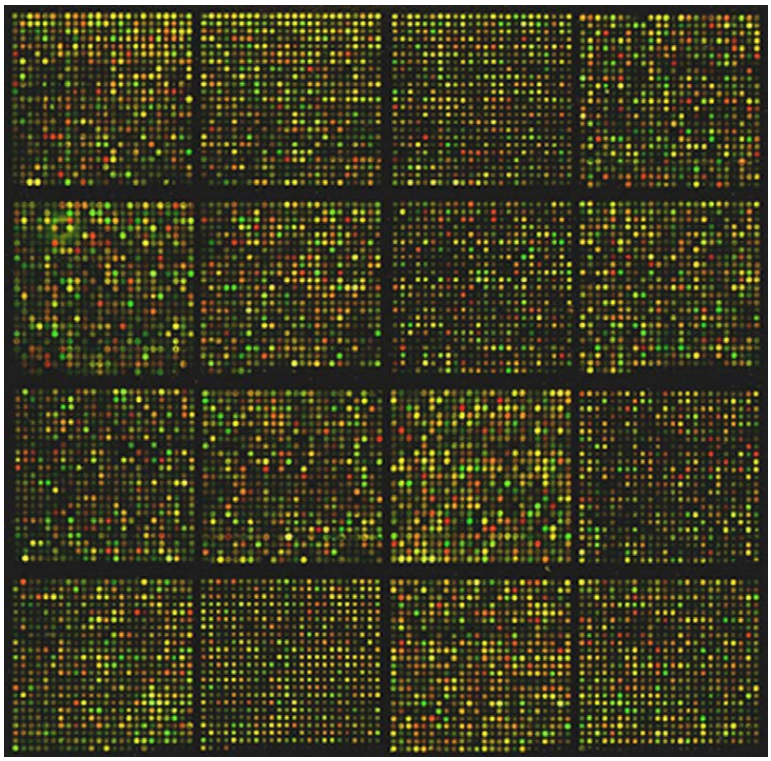


Figure 8.23 - Microarray design. Image by Taralyn Tan

To the slide are added a mixture of sample molecules, some of which will recognize and bind specifically to the sequences on the slide. Binding between the sample molecules and the sequences attached to the slide occurs by base pairing, in the case of DNA microarrays. The slide is then washed to remove sample molecules that are not specifically bound to the sequences in the grid.

Sample molecules are tagged with a fluorescent dye, allowing the spots where they bind to be identified. The grid is analyzed spot by spot for binding of the sample molecules to the immobilized sequences. The more sample molecules are bound at a spot, the greater the intensity of dye fluorescence that will be observed. Information from this analysis can give information about the presence/absence/abundance of molecules in the sample that bind to the sequences in the grid.

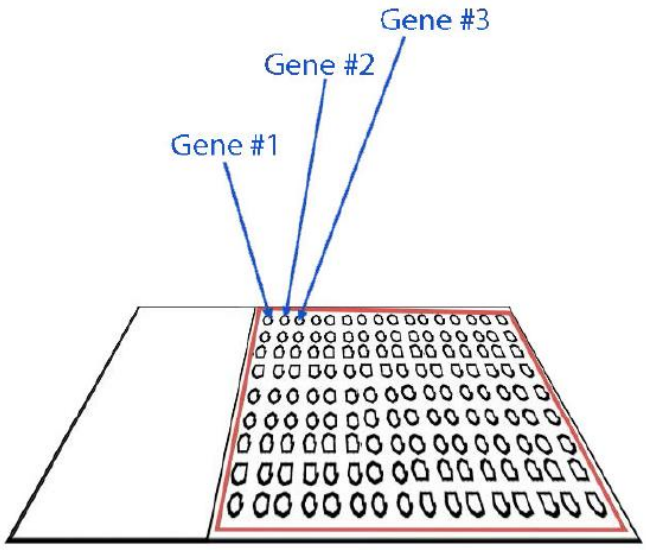


Figure 8.24 - Large scale microarray analysis of mouse transcriptome. Wikipedia

This page titled 9.4: Detection, identification and quantitation of specific nucleic acids and proteins is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

https://chem.libretexts.org/@go/page/347470

9.5: Transcriptomics

Consider a matrix containing all of the known gene sequences in a genome. To make such a matrix for analysis, one would need to make copies of every gene, either by chemical synthesis or by using the polymerase chain reaction. The strands of the resulting DNAs would then be separated to obtain single-stranded sequences that could be attached to the chip. Each box of the grid would contain sequence from one gene. With this grid, one could analyze the transcriptome - all of the mRNAs being made in selected cells at a given time. For a simple analysis, one could take a tissue (say liver) and extract all the mRNAs from it. This mRNA population represents all the genes that were being expressed in the liver cells at the time the RNA was extracted. These RNAs should be able to hybridize (base-pair) with their corresponding genes on the microarray. Genes that were not being expressed would have no mRNAs to bind to their corresponding genes on the grid.

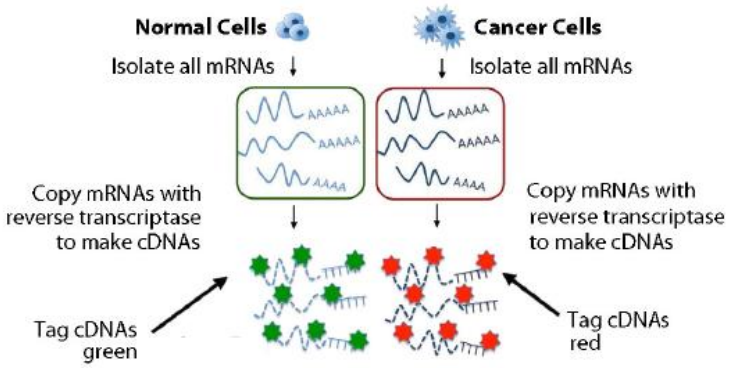


Figure 8.25 - Copying and labeling of transcriptome. Image by Taralyn Tan

In practice, the mRNAs are not used directly, but are copied into single-stranded DNA copies called cDNAs. The cDNAs are tagged with a fluorescent dye and added to the microarray under conditions that allow base pairing so that the cDNAs can find and base pair with complementary sequences on the matrix (Figure 8.26). The matrix is then washed to remove unhybridized cDNAs. The presence/absence/abundance of each mRNA is then readily determined by measuring the amount of dye at each box of the grid.

Figure 8.26 - Add labeled cDNAs to microarray plate. Image by Taralyn Tan

In Figure 8.27, a fluorescent cDNA has bound to the spot on the far right in the third row of the grid. This means that the sequence of the cDNA was complementary to the sequence of the gene sequence immobilized at that spot. Because the identity of the genes at each position on the grid is known, we then know that the sample contained mRNA that corresponded to that particular gene. In other words, that gene was being expressed in the cells from which the mRNAs were obtained.

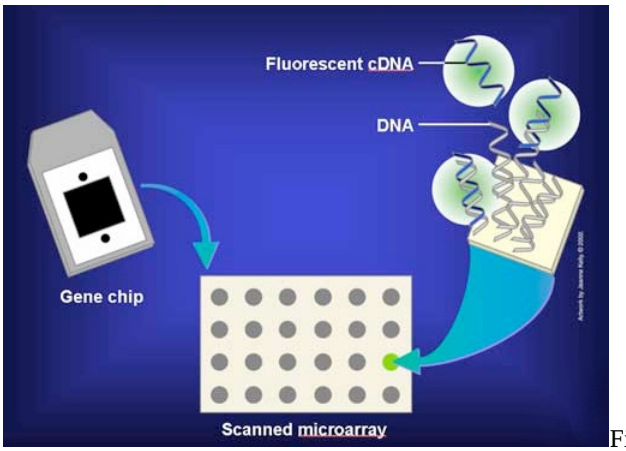


Figure 8.27 - Binding of a fluorescent cDNA copy of a specific mRNA to DNA immobilized on one spot in a microarray. Wikipedia

A more powerful analysis could be performed with two sets of mRNAs simultaneously. . One set of cDNAs could come from a cancerous tissue and the other from a non-cancerous tissue, for example. The cDNAs derived from each sample is marked with a

different color (say green for normal and red for cancerous) (Figure 8.25). The cDNAs are mixed and then added to the matrix and complementary sequences are once again allowed to form duplexes (Figure 8.27).

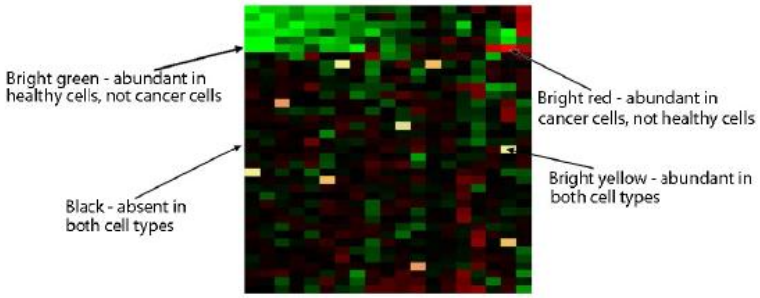


Figure 8.28 - Microarray analysis comparing gene expression in normal and cancer cells. Wikipedia

Unhybridized cDNAs are washed away and then the plate is analyzed. Red grid boxes correspond to an mRNA present in the cancerous tissue, but not in the non-cancerous tissue. Green grid boxes correspond to an mRNA present in the non-cancerous tissue, but not in the cancerous tissue. Yellow would correspond to mRNAs present in equal abundance in the two tissues (Figure 8.28). The intensity of each spot also gives information about the relative amounts of each mRNA in each tissue.

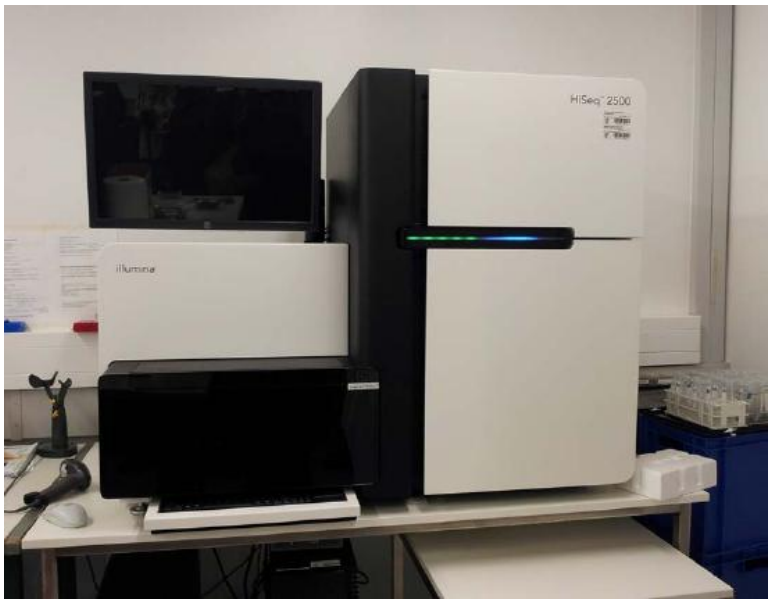


Figure 8.29 - Automated high throughput sequencer. Wikipedia

The same principle used for nucleic acid microarrays can be adapted for analyzing other molecules. For example, polypeptides could be bonded to the glass slide instead of DNA to create a protein chip. Protein chips are useful for studying the interactions of proteins with other molecules as well as for diagnostics.

RNA-Seq Technique

Like microarrays, a newer method called RNA-Seq, is a tool for simultaneously detecting and quantitating all of the transcripts in a given sample. This method relies on recently developed sequencing technologies called next-generation sequencing, or deep sequencing. These techniques allow for rapid, parallel sequencing of millions of DNA fragments and can, thus, be used not only for genomic DNA, but also to sequence all of the reverse-transcribed RNAs from a given sample.

To determine all the protein-coding genes that were being expressed in a particular set of cells under specific physiological conditions, all of the mRNA would first be extracted and reverse-transcribed into cDNA. This step is similar to the preparation of samples for microarrays. However, at this point, the cDNAs are fragmented into smaller pieces, and have small sequencing adapters attached at either end. The fragments are then subjected to high-throughput sequencing, to obtain short sequences from all of the fragments. These data are aligned against the genome sequence and used to measure the level of expression of different

genes. RNA-Seq offers some advantages over microarrays. With microarrays, an RNA can only be detected if the gene sequence corresponding to it is present on the grid. In RNA-Seq every RNA present in the sample is sequenced, so detection of RNAs is not limited by the probes on a chip. RNA-Seq is more sensitive than microarrays and offers a much larger range over which gene expression can be measured accurately.

9.5: Transcriptomics is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by LibreTexts.

- **8.5: Transcriptomics** is licensed CC BY-NC-SA 4.0.

9.6: Isolating Genes

Earlier in this chapter, we discussed methods such as column chromatography that are used to purify proteins of interest. Using combinations of these methods, it is possible to isolate a protein to a high degree of purity, thus enabling us to study the protein's activity and properties. This problem is harder to solve for nucleic acids. Genomic DNA can be readily obtained from cells, but is too complex to be analyzed as a whole. Individual genes are the units of DNA that correspond to proteins, and thus, it makes more sense to isolate specific genes for study. Methods to isolate genes were not available till the 1970s, when the discovery of restriction enzymes and the invention of molecular cloning provided, for the first time, ways to obtain large quantities of specific DNA fragments, for study. Although, for purposes of obtaining large amounts of a specific DNA fragment, molecular cloning has been largely replaced by direct amplification using the polymerase chain reaction described later, cloned DNAs are still very useful for a variety of reasons. The development of molecular cloning was dependent on the discovery of restriction endonucleases, described below.

Restriction enzymes

Restriction enzymes, or restriction endonucleases, are enzymes made by bacteria. These enzymes protect bacteria by degrading foreign DNA molecules that are carried into their cells by, for example, an invading bacteriophage. Each restriction enzyme recognizes a specific sequence, usually of four or six nucleotides in the DNA. These sequences, when they occur in the bacterium's own DNA, are chemically modified by methylation, so that they are not recognized and degraded. Where these sequences occur in foreign DNA, they are cut by the restriction enzyme.

The utility and importance of restriction enzymes lies in their ability to recognize specific sequences in DNA and cut near or (usually) at the site they recognize. Over 3000 such enzymes are known. Sequences recognized by these enzymes are typically 4-8 base pairs long and the most commonly used enzymes recognize sequences described as palindromic.

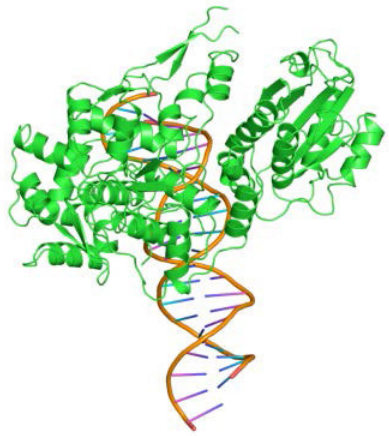


Figure* 9.6.1*: *-A restriction enzyme bound to its recognition sequence on DNA. Wikipedia*

Palindrome

In molecular biology, the term palindrome means that the sequence of the recognition site when read in the 5‘ to 3‘ direction for the top strand is exactly the same as that of the bottom strand. Consider the sequence recognized by the restriction enzyme known as Hind III (pronounced hin-dee-three). It is

5’ -A-A-G-C-T-T-3’
3’ -T-T-C-G-A-A-5’

On the top strand, the recognition sequence is

5’ AAGCTT 3’

which is the same as the bottom strand (read in the same 5’ to 3’ direction).

While all restriction enzymes must recognize and bind to particular DNA sequences, the exact spot at which they cut the DNA varies. Some enzymes leave a staggered sequence after cutting that has an overhang at the 5' end of one strand of the duplex; some leave a staggered sequence after cutting that has an overhang at the 3' end; and some cut both strands in the same place, leaving no overhanging sequence - called blunt end cutters.

Consider cutting a DNA sequence that contains the Hind III recognition site, which is

5' -A-A-G-C-T-T-3'
3' -T-T-C-G-A-A-5'

Embedded within a DNA sequence, the Hind III sequence would look like this (Ns correspond to any base and represent all of the DNA around the recognition site).

5' -N-N-N-A-A-G-C-T-T-N-N-N-3'
3' -N-N-N-T-T-C-G-A-A-N-N-N-5'

After cutting with Hind III, it would look as follows:

5' -N-N-N-A 3' 5'A-G-C-T-T-N-N-N-N-3'
3' -N-N-N-T-T-C-G-A-5' 3' A-N-N-N-N-5'

where gaps have been inserted to illustrate where cutting has occurred. Hind III cuts between the two 'A' containing nucleotides near the 5' end of the recognition sequence and thus leaves 5' overhangs (Figure **9.6.2**).

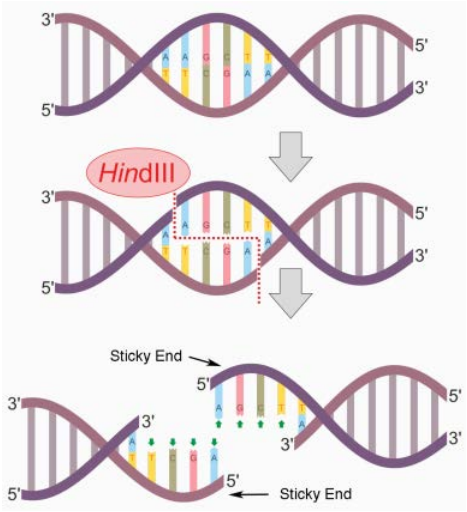


Figure* 9.6.2*: *Result of cutting DNA with Hind III. Wikipedia*

The restriction enzyme Pst I, on the other hand, recognizes the following sequence

5' -N-N-N-C-T-G-C-A-G-N-N-N-N-3'
3' -N-N-N-G-A-C-G-T-C-N-N-N-N-5'

and cuts between the A and the G near the 3' end of the recognition sequence.

5' -N-N-N-C-T-G-C-A 3' 5'G-N-N-N-N 3'
3' -N-N-N-G 5' 3' A-C-G-T-C-N-N-N-N 5'

As you can see, cutting a DNA with Pst I leaves 3' overhangs of the recognition sequence. The ends left after cutting by a restriction enzyme that overhang either at the 5' end or the 3' end are referred to as being "sticky" because they can form proper base pairs and more readily be joined to a similarly "sticky end". This means that you can take two unrelated pieces of DNA, cut them with the same restriction enzyme so that they have compatible sticky ends, and then "paste" them together using DNA ligase to form a new hybrid molecule, or recombinant.

Making Recombinant DNAs

Joining together of DNA fragments from different sources creates recombinant DNA. The ability to cut and paste DNA might seem like purely a technical feat, but one key application that arose out of this is molecular cloning. In molecular cloning a gene of interest can be inserted into a vector, usually a plasmid, by cutting both the vector and the gene (called the insert) with the same enzyme to generate sticky ends and joining the two pieces together to generate a recombinant (Figure **9.6.3**). A plasmid is a type of autonomously replicating, extrachromosomal DNA. It is quite simple to extract plasmids from the cells, engineer them to contain the gene of interest and re-introduce the recombinant plasmid into the bacteria. The idea was that when the plasmid DNA was replicated, the extra inserted gene would also be copied. Thus, by growing up a lot of the bacteria carrying the plasmid, many copies of the gene of interest could be obtained, to provide sufficient amounts of the gene to use in experiments. While we now have easier methods to accomplish this goal, cloned DNAs remain very useful. For example, it is possible to clone a gene that encodes a protein of interest so that it can be expressed at high levels in the cells into which the recombinant plasmid is introduced.

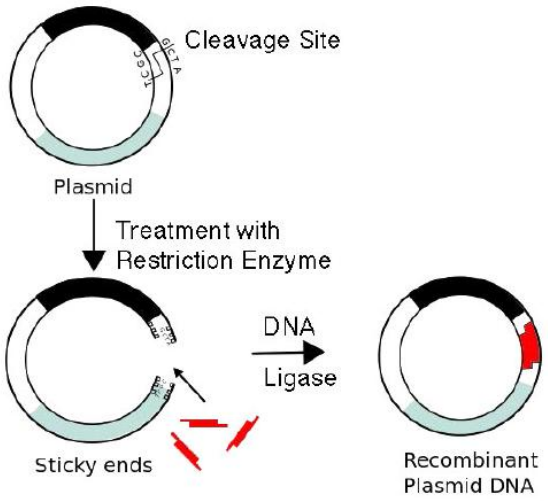


Figure* 9.6.3: Recombinant DNA construction. Wikipedia*

Whatever the purpose for which the recombinant plasmid is made, it typically carries an antibiotic resistance gene (or genes), called a selectable marker. Cells that take up the plasmid will be able to grow in the presence of the antibiotic. If bacterial cells to which the plasmid has been added are plated on agar containing the antibiotic, the cells which took up the plasmid will be able to grow, while the others will not.

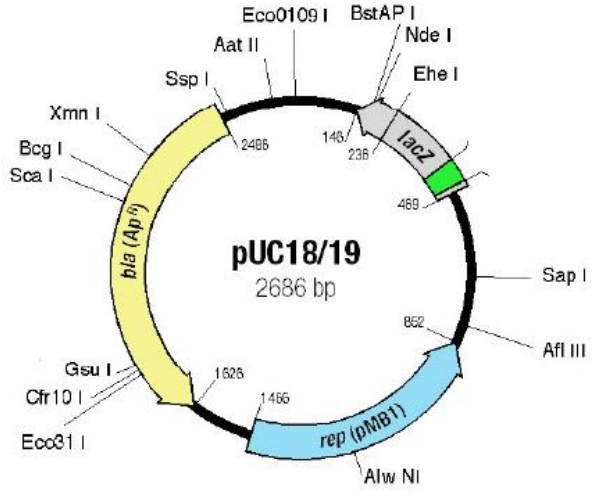


Figure **9.6.4**: Restriction site map for the pUC 18/19 plasmids, a classic plasmid vector. Genes identified by arrows. Numbers correspond to the pUC 18/19 numbering convention

Expression cloning

As mentioned above, a gene of interest may be inserted into a vector and the recombinant plasmid be placed into a cell where the gene can be expressed. For instance, one might desire to clone the gene coding for human growth hormone or insulin or other medically important proteins and have a bacterium or yeast make large quantities of it very cheaply. Remember that these are human proteins, and thus it is not feasible to extract the proteins in any quantity from human subjects.

To clone a gene so that it can be expressed, one needs to set up the proper conditions in order for the human protein to be made in the bacterial cells. This typically involves the use of specially designed plasmids. These plasmids have been engineered to 1) replicate in high numbers; 2) carry markers that allow researchers to identify cells carrying them (antibiotic resistance, for example) and 3) contain sequences (such as a promoter and Shine Dalgarno sequence) necessary for expression of the desired protein, with convenient sites for insertion of the gene of interest in the appropriate place relative to the control sequences. A plasmid which has all of these features is referred to as an expression vector. In addition to plasmids that can be used for expression in bacterial cells, expression vectors are also available that allow protein expression in a variety of eukaryotic cells.

Many sophisticated variations on such vectors have been created that have made it easy to produce and purify large amounts of any protein of interest for which the gene has been cloned. A handy feature in some expression vectors is a sequence encoding an affinity tag either up- or downstream of the gene being expressed. This sequence allows a short affinity tag (such as a run of histidine residues) to be fused onto the encoded protein. The tag can be used to readily purify the protein, as described in the section on affinity chromatography.

This page titled 9.6: Isolating Genes is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **8.6: Isolating Genes** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

9.7: Polymerase Chain Reaction (PCR)

Molecular cloning was the first method available to isolate a gene of interest and make many copies of it to obtain sufficient amounts of the DNA to study. Today, there is a faster and easier way to obtain large amounts of a DNA sequence of interest -the polymerase chain reaction (PCR). PCR allows one to use the power of DNA replication to amplify DNA enormously in a short period of time. As you know, cells replicate their DNA before they divide, and in doing so, double the amount of the cell's DNA. PCR essentially mimics cellular DNA replication in the test tube, repeatedly copying the target DNA over and over, to produce large quantities of the desired DNA.

Selective Replication

In contrast to cellular DNA replication, which amplifies all of a cell's DNA during a replication cycle, PCR does targeted amplification to replicate only a segment of DNA bounded by the two primers that determine where DNA polymerase begins replication. Figure 8.34 illustrates the process. Each cycle of PCR involves three steps, denaturing, annealing and extension, each of which occurs at a different temperature.

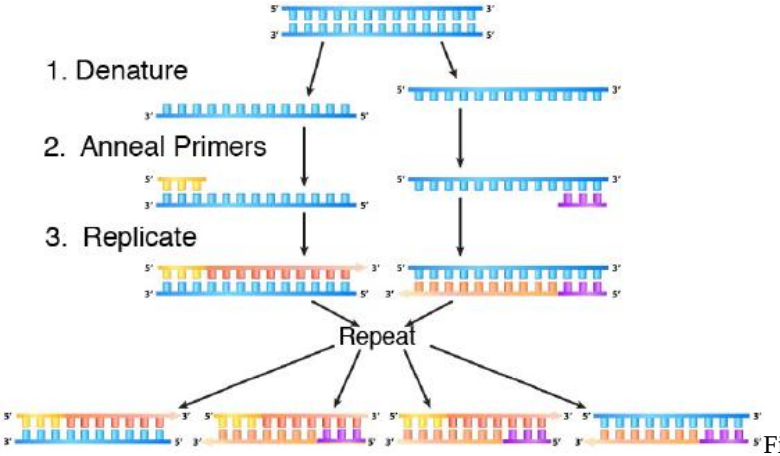


Figure 8.34 - Steps in the polymerase chain reaction. Image by Aleia Kim

The Starting Materials

Since PCR is, basically, replication of DNA in a test-tube, all the usual ingredients needed for DNA replication are required:

- A template (the DNA containing the target sequence that is being copied)
- Primers (to initiate the synthesis of the new DNA strands)
- Thermostable DNA polymerase (to carry out the synthesis). The polymerase needs to be heat stable, because heat is used to separate the template DNA strands in each cycle.
- dNTPs (DNA nucleotides to build the new DNA strands).
- The template is the DNA that contains the target you want to amplify (the "target" is the specific region of the DNA you want to amplify).

The primers are short synthetic single-stranded DNA molecules whose sequence matches a region flanking the target sequence. It is possible to chemically synthesize DNA molecules of any given base sequence, to use as primers. To make primers of the correct sequence that will bind to the template DNA, it is necessary to know a little bit of the template sequence on either side of the region of DNA to be amplified. DNA polymerases and dNTPs are commercially available from biotechnology supply companies.

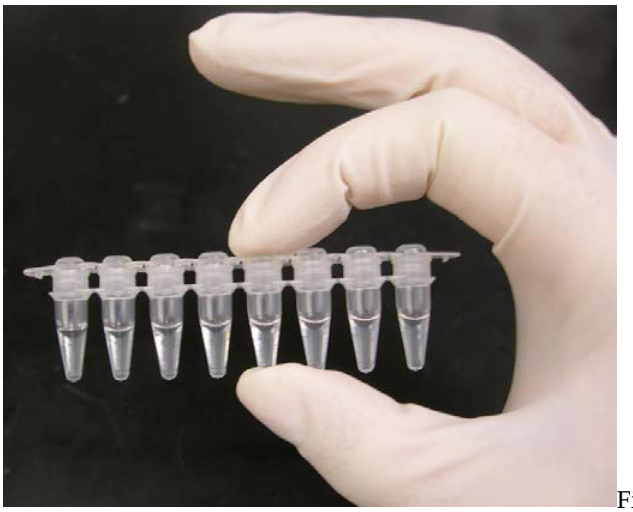


Figure 8.35 - PCR tubes with DNA samples ready for reaction. Wikipedia

First, all of the reagents are mixed together. Primers are present in millions of fold excess over the template. This is important because each newly made DNA strand starts from a primer. The first step of the process involves separating the strands of the target DNA by heating to near boiling.

Next, the solution is cooled to a temperature that favors complementary DNA sequences finding each other and making base pairs, a process called annealing. Since the primers are present in great excess, the complementary sequences they target are readily found and base-paired to the primers. These primers direct the synthesis of DNA. Only where a primer anneals to a DNA strand will replication occur, since DNA polymerases require a primer to begin synthesis of a new strand.

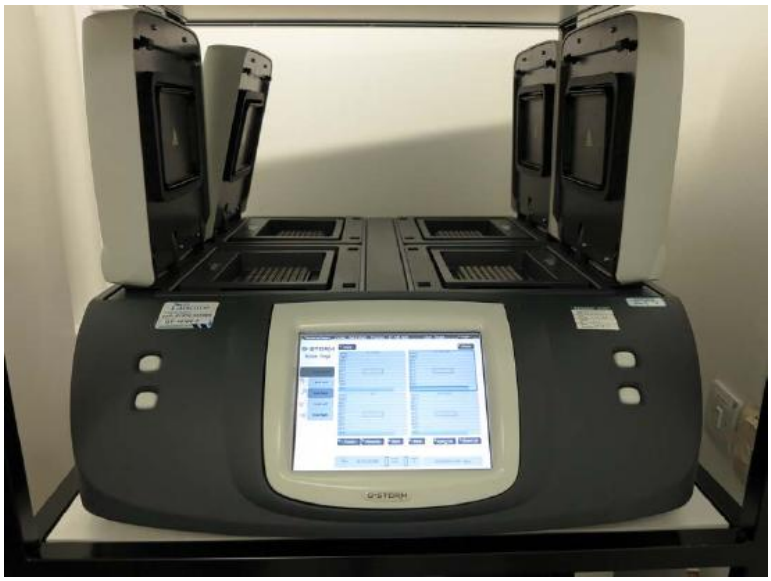


Figure 8.36 - A PCR thermocycler system. Wikipedia

Extension

In the third step in the process, the DNA polymerase replicates DNA by extension from the 3’ end of the primer, making a new DNA strand. At the end of the first cycle, there are twice as many DNA molecules, just as in cellular replication. But in PCR, the process is repeated, usually for between 25 and 30 cycles. At the end of the process, there is a theoretical yield of 230 (over 1 billion times) more DNA than there was to start. (This enormous amplification power is the reason that PCR is so useful for forensic investigations, where very tiny amounts of DNA may be available at a crime scene.)

The temperature cycles are controlled in a thermocycler, which repeatedly raises and lowers temperatures according to the set program. Since each cycle can be completed in a couple of minutes, the entire amplification can be completed very rapidly. The

resulting DNA is analyzed on a gel to ensure that it is of the expected size, and depending on what it is to be used for, may also be sequenced, to be certain that it is the desired fragment.

Mutagenesis

PCR is frequently used to obtain gene sequences to be cloned into vectors for protein expression, for example. Besides simplicity and speed, PCR also has other advantages. Because primers can be synthesized that differ from the template sequence at any given position, it is possible to use PCR for site-directed mutagenesis. That is, PCR can be used to mutate a gene at a desired position in the sequence. This allows the proteins encoded by the normal and mutant genes to be expressed, purified and compared.

Analysis of gene expression

PCR can also be used to measure gene expression. Where in PCR the amount of amplified product is not determined till the end of all the cycles, a variation called quantitative real-time PCR is used, in which the accumulation of product is measured at each cycle. This is possible because real-time PCR machines have a detector module that can measure the levels of a fluorescent marker in the reaction, with the amount of fluorescence proportional to the amount of amplified product. By following the accumulation of product over the cycles it is possible to calculate the amount of starting template. To measure gene expression, the template used is mRNA reverse-transcribed into cDNA (see below). This coupling of reverse transcription with quantitative real-time PCR is called qRT-PCR.

This page titled 9.7: Polymerase Chain Reaction (PCR) is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **8.7: Polymerase Chain Reaction (PCR)** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

9.8: Reverse Transcription

In the central dogma, DNA codes for mRNA, which codes for protein. One known exception to the central dogma is exhibited by retroviruses. These RNA-encoded viruses have a phase in their life cycle in which their genomic RNA is converted back to DNA by a virally-encoded enzyme known as reverse transcriptase. The ability to convert RNA to DNA is a method that is desirable in the laboratory for numerous reasons. For example, converting RNAs of interest to cDNA is used in RT-PCR as well as in other applications like microarray analysis.

Process

First, one creates a DNA oligonucleotide to serve as a primer for reverse transcriptase to use on a target RNA. The primer must, of course, be complementary to a segment (near the 3' end) of the RNA to be amplified. The RNA, reverse transcriptase, the primer, and four dNTPs are mixed. With one round of replication, the RNA is converted to a single strand of DNA. Denaturation frees the single stranded cDNA, which can be used as is, or converted to double-stranded cDNA, depending on the application.

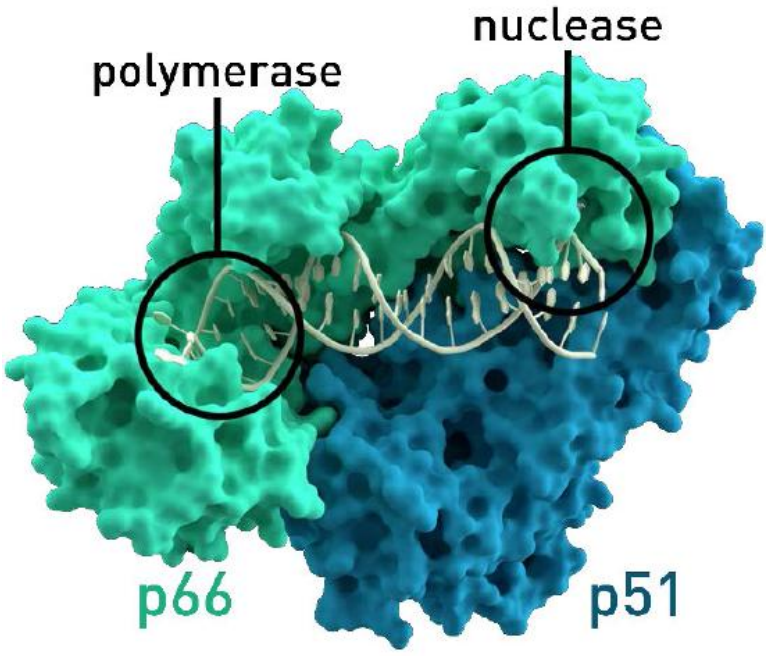


Figure 8.37 - Reverse transcriptase of HIV. The nuclease function is needed for the viral life cycle, but not for lab use. Wikipedia

Detecting molecular interactions

The study of biochemistry is basically the study of the interactions of cellular molecules. Methods for detecting interactions among biomolecules are, for this reason, very useful to biochemists. We will now discuss a couple of very different methods for detecting these inter-molecular interactions.

Yeast two-hybrid system (Y2H)



Figure 8.38 - Structure of a eukaryotic transcriptional activator, showing the DNA-binding domain (DBD) and transcriptional activation domain (TAD). Wikipedia

Yeast two-hybrid screening is a sophisticated technique for identifying which protein(s), out of a collection of all of a cell's proteins, interacts with a specific protein of interest. The method relies on the interaction between two proteins to reconstitute a

functional transcriptional activator within yeast cells. You may remember that many transcriptional activators are modular proteins that have a domain that binds to DNA and another domain that activates transcription (Figure 8.38).

If the transcription factor is split, so that the binding domain is attached to one protein, and the activation domain to another protein, a functional transcriptional activator can only be re-created if the two “carrier”proteins come into close proximity - that is, they interact. The presence of this functional activator can be detected by the expression of a reporter gene.

A simple way to understand this idea is by thinking of a transcriptional activator as a device, like a flashlight, that has two parts, the battery and the lamp, that must be together in order to function. Neither a person who has just a battery nor one who has only the lamp will be able to see in a dark room. But if the two interact by coming close enough to insert the battery in the flashlight, their interaction can be detected by the fact that the flashlight will now be functional as evidenced by the light produced.

It takes two to tango

Figure 8.39 (A) shows the normal yeast transcriptional activator, GAL4, with both the DNA-binding (DBD) and Activation domains (AD). It is able to stimulate transcription of the downstream reporter gene, lac z. Panels B and C show constructs that produce the GAL4 DBD and AD, respectively, fused to other proteins, one of which is termed the “bait” and the other as “prey”. Neither of these fusion proteins can stimulate transcription of the lac z gene. When constructs encoding both the bait and prey are in the same yeast cell, if the bait protein interacts with the prey, the DBD and AD of the GAL4 will be brought together to reconstitute a functional GAL4. The presence of functional GAL4 is readily detectable because it will stimulate expression of the lac z reporter gene. If the bait and prey proteins do not interact, then there will be no lac z expression. When interaction is detected through expression of the reporter gene, the specific prey protein can then be identified.

The yeast two-hybrid system allows for simultaneous screening of many prey proteins, by constructing large collections of fusion constructs, with each potential protein partner of the bait protein fused to the GAL4 activation domain.

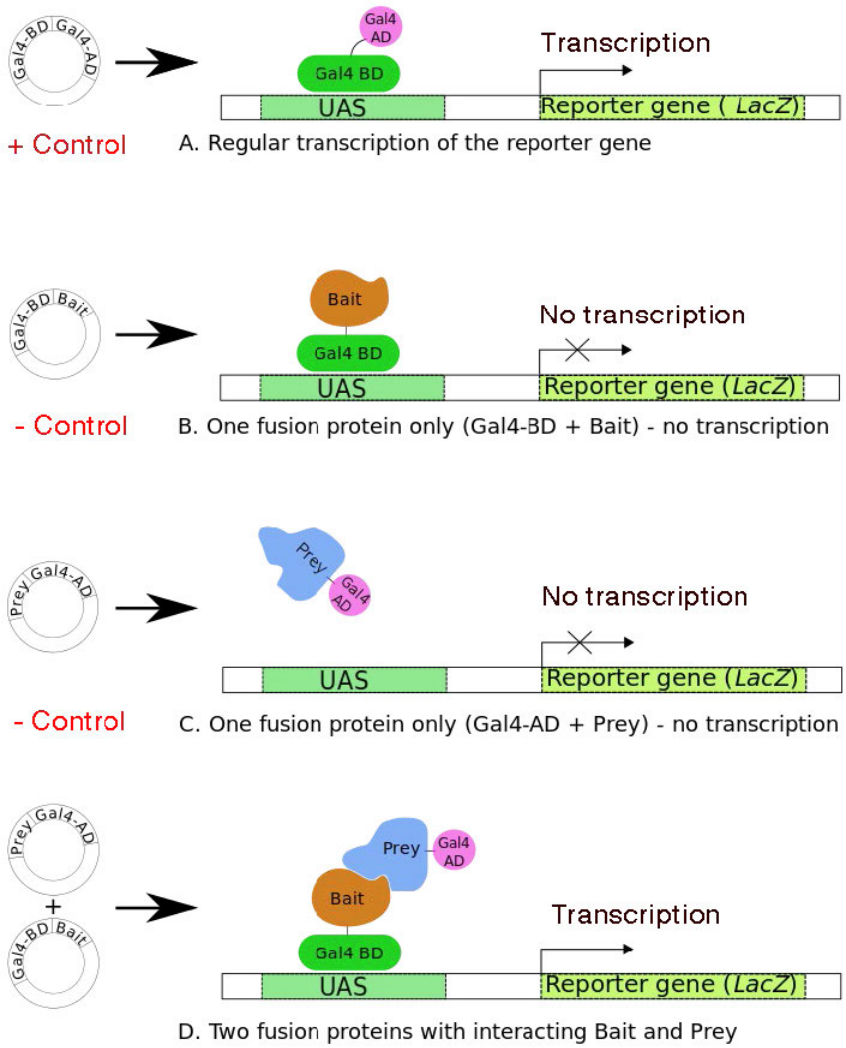


Figure 8.39 - Four scenarios for the yeast two-hybrid system. UAS = Upstream Activator Sequences - acts like a promoter. Scenario A shows that the two transcription factors start out as one protein. Wikipedia

---

This page titled 9.8: Reverse Transcription is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **8.8: Reverse Transcription** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

9.9: Genome Editing (CRISPR)

The development of tools that would allow scientists to make specific, targeted changes in the genome has been the Holy Grail of molecular biology. An ingenious new tool that is both simple and effective in making precise changes is poised to revolutionize the field, much as PCR did in the 1980s. Known as the CRISPR/Cas9 system, and often abbreviated simply as CRISPR, it is based on a sort of bacterial immune system that allows bacteria to recognize and inactivate viral invaders.

CRSPR

CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats, short repeated sequences found in prokaryotic DNA, separated by spacer sequences derived from past encounters with, for example, a bacteriophage. Like the glass slipper left behind by Cinderella that was later used to identify her, the pieces of the invader's sequences are a way for the bacteria to identify the virus if it attacks again. Inserted into the bacterial genome, these sequences can later be transcribed into a guide RNA that matches, and base-pairs with, sections of the viral genome if it was encountered again. A nuclease associated with the guide RNA then cleaves the sequence base-paired with the guide RNA. (The nucleases are named Cas for CRISPR-associated.)

The essential elements of this system are a guide RNA that homes in on the target sequence and a nuclease that can make a cut in the sequence that is bound by the guide RNA. By engineering guide RNAs complementary to a target gene, it is possible to target the nuclease to cleave within that gene. In the CRISPR/Cas9 system, the Cas9 endonuclease cuts both strands of the gene sequence targeted by the guide RNA (Figure **9.9.1**). This generates a double-strand break that the cell attempts to repair.

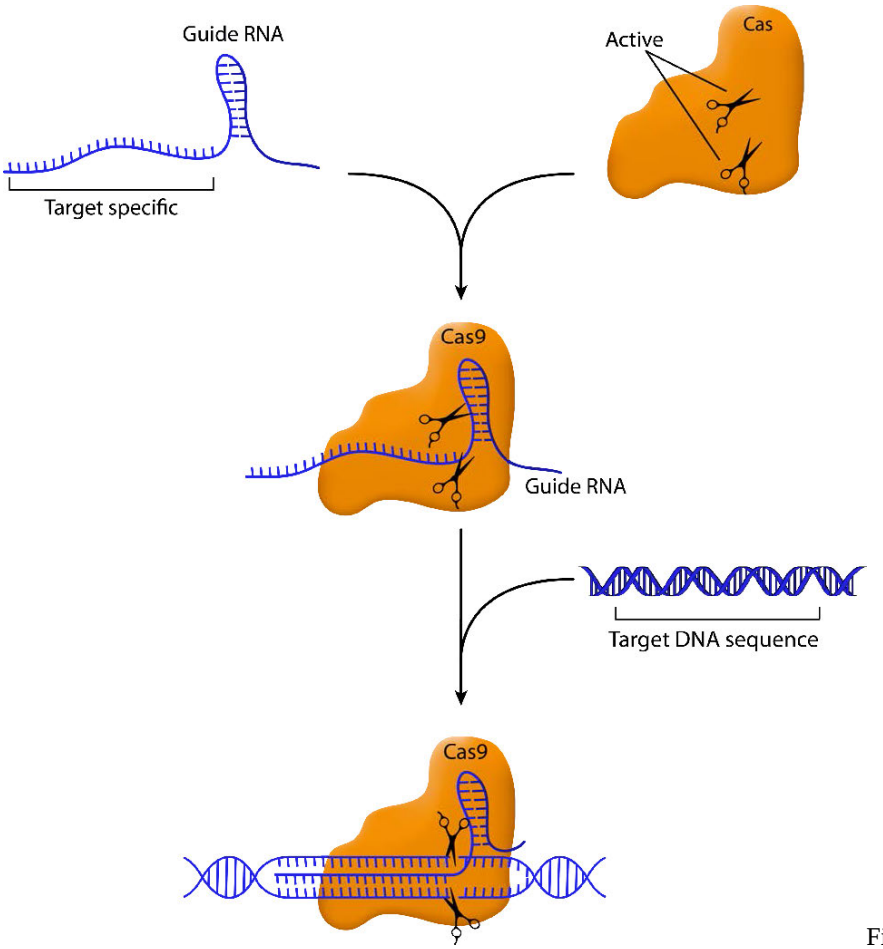


Figure **9.9.1**: A guide RNA directs the Cas9 nuclease to its target gene. Image by Pehr Jacobsen

As you may remember, double-strand breaks in DNA can be repaired by simple, nonhomologous end joining (NHEJ) or by homologous recombination. When a break is fixed by NHEJ, there is good chance that there will be deletions or insertions that will

inactivate the gene they are in. Thus, targeted cleavage of a site by CRISPR/Cas9 can easily and specifically inactivate a gene, making it easy to characterize the gene's function.

But, what if you wished to simply mutate the gene at a specific site to study the effect of the mutation? This, too, can be achieved. If a homologous sequence bearing the specific mutation is provided, homologous recombination can repair the break, and at the same time insert the exact mutation desired. It is obvious that if you can insert a mutation as just described, it should be possible to correct a mutation in the genome by cleaving at the appropriate spot and providing the correct sequence as a template for repair by homologous recombination. The simplicity of the system holds great promise for curing genetic diseases.

Scientists have also come up with some creative variations on the CRISPR/Cas9 system. For instance, one variant inactivates the nuclease activity of Cas9. The guide RNA in this system pairs with the target sequence, but the Cas9 does not cleave it. Instead, the Cas9 blocks the transcription of the downstream gene (Figure **9.9.2**) This method allows specific genes to be turned off without actually altering the DNA sequence.

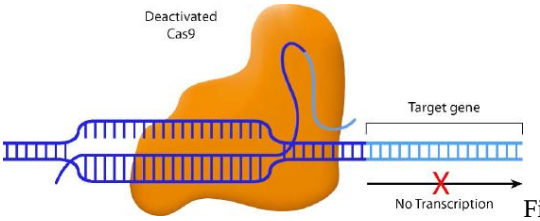


Figure **9.9.2**: Inactive Cas9 can block transcription of a target gene. Image by Pehr Jacobsen

Another variation also uses a disabled Cas9, but this time, the Cas9 is fused to a transcriptional activation domain. In this situation, the guide RNA positions the Cas9-activator domain in a place where it can enhance transcription from a specific promoter (Figure **9.9.3**). Other variations on this theme attach histone-modifying enzymes or DNA methylases to the inactive Cas9. Again, the guide RNA positions the Cas9 in the desired spot, and the enzyme attached to Cas9 can methylate the DNA or modify the histones in that region.

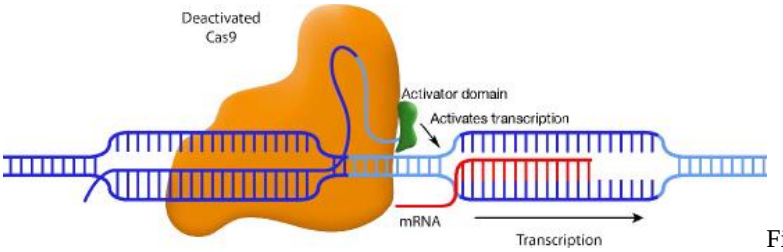


Figure **9.9.3**: A Cas9-activator domain fusion can activate transcription of a target gene. Image by Pehr Jacobsen

CRISPR has already been used to edit genomes in a wide variety of species (and in human cell cultures). It may not be long before the technique is approved for clinical use. In the meanwhile, CRISPR is transforming molecular biology.

This page titled 9.9: Genome Editing (CRISPR) is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

9.10: Membrane Dynamics (FRAP)

Understanding the dynamics of movement in the membranes of cells is the province of the Fluorescence Recovery After Photobleaching (FRAP) technique (Figure 8.47). This optical technique is used to measure the two dimensional lateral diffusion of molecules in thin films, like membranes, using fluorescently labeled probes. It also has applications in protein binding.

In the method, a lipid bilayer is uniformly labeled with a fluorescent tag (Figure 8.47, Step A) and then a subset of the tag is bleached using a laser (Step B). The spread of the bleached molecules is followed using a microscope (Step C). Information obtained in this manner provides data about the rate of lateral diffusion occurring in a lipid bilayer (Step D).

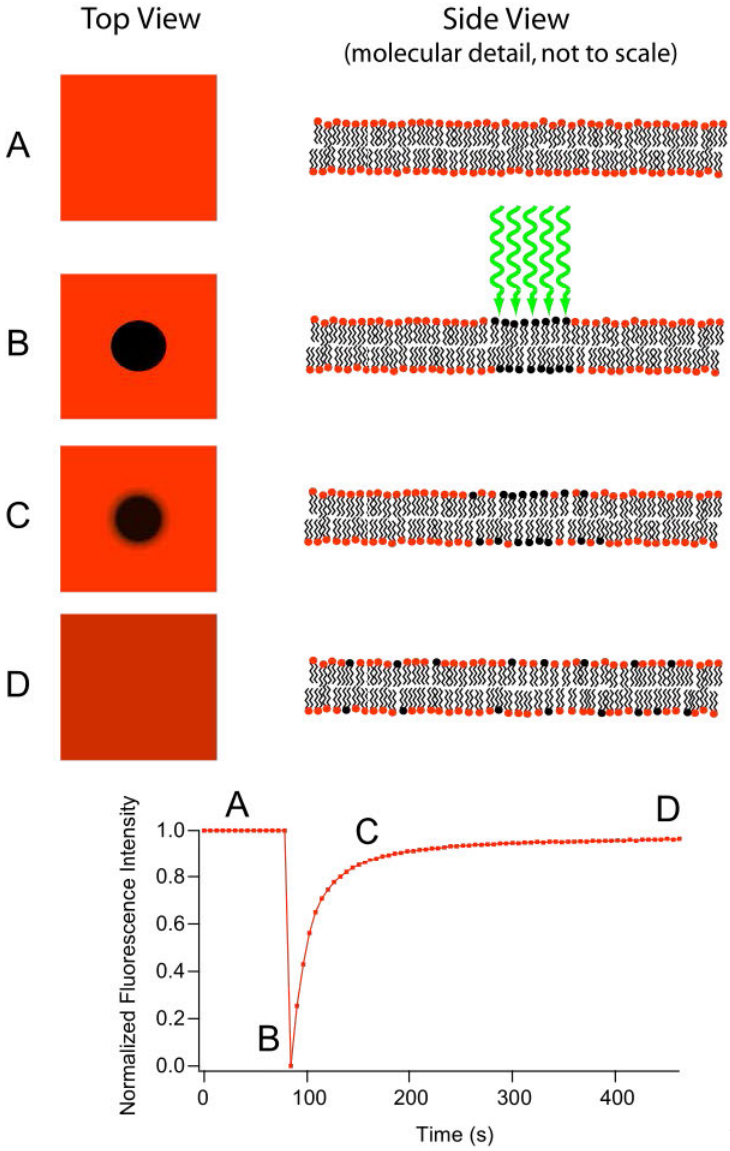


Figure 8.47 - FRAP analysis. Wikipedia

This page titled 9.10: Membrane Dynamics (FRAP) is shared under a CC BY-NC-SA 4.0 license and was authored, remixed, and/or curated by Kevin Ahern, Indira Rajagopal, & Taralyn Tan.

- **8.12: Membrane Dynamics (FRAP)** by Kevin Ahern, Indira Rajagopal, & Taralyn Tan is licensed CC BY-NC-SA 4.0. Original source: http://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy.

CHAPTER OVERVIEW

10: Supplemental Modules (Biochemistry)

http://www.mikeblaber.org/oldwine/BC...l/bch4053l.htm -lectur notes

http://www.mikeblaber.org/oldwine/bch5425/bch5425.htm

10.1: DNA

10.1.1: DNA as Genetic Material

10.1.2: Structure of DNA and RNA

10.1.3: Bacterial Restriction/Modification system

10.1.4: DNA Modifying Enzymes

10.1.5: DNA Replication - Introduction to Prokaryotic replication

10.1.6: DNA Supercoiling and Topoisomerases

10.2: Bacteria

10.2.1: The Bacteria - An Introduction

10.2.2: Bacterial Growth and Reproduction

10.2.3: Extrachromosomal elements, plasmids, selectable markers

10.2.4: Central Dogma and the Genetic Code

10.2.5: Gene and Operon

10.2.6: The lac Operon; CAP site; DNA footprinting

10.2.7: Transcriptional Regulation- Transcription termination, the trp operon

10.2.8: mRNA Translation

10.3: 3. Biotechnology 1

10.3.1: Gel Electrophoresis

10.3.2: DNA Sequence Analysis

10.3.3: Polymerase Chain Reaction (PCR) and Cloning of PCR Products

10.3.4: Prokaryotic Expression Vectors

10.3.5: Protein Sequencing, Peptide Mapping, Synthetic Genes

10.3.6: cDNA and Genomic Libraries

10.3.7: Protein Purification

10.4: 5. Lab Notes Part 1

10.4.1: Basic practices and techniques, Laboratory notebooks, Presentation of data, Sections of a scientific report

10.4.2: Spectrophotometry

10.4.3: Quantification of Protein Concentration

10.4.4: Chromatography

10.4.5: Gel Electrophoresis of Proteins

10.4.6: Introduction to Protein Purification

10.5: 6. Lab Notes Part 2

10.5.1: Genetic Transformation (using bacteria and the pGLO plasmid)

10.5.2: Enzyme kinetics

10.5.3: Ligand binding

10.5.4: Restriction Mapping

10.5.5: Polymerase Chain Reaction (PCR)

10.5.6: Use of PC and internet for biochemical research
10.6: 4. Biotechnology 2
10.6.1: M13 Phage
10.6.2: M13 Phage Display Libraries
10.6.3: SELEX (Selective Evolution of Ligands by Exponential Enrichment)
10.6.4: Protein-Protein Recognition Probed Using a Yeast Transcriptional Activator System
10.6.5: Molecular Imprinting

This page titled 10: Supplemental Modules (Biochemistry) is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

SECTION OVERVIEW

10.1: DNA

10.1.1: DNA as Genetic Material

10.1.2: Structure of DNA and RNA

10.1.3: Bacterial Restriction/Modification system

10.1.4: DNA Modifying Enzymes

10.1.5: DNA Replication - Introduction to Prokaryotic replication

10.1.6: DNA Supercoiling and Topoisomerases

This page titled 10.1: DNA is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

10.1.1: DNA as Genetic Material

In the early 1900's many people thought that **protein** must be the **genetic material** responsible for inherited characteristics. One of the reasons behind this belief was the knowledge that proteins were quite complex molecules and therefore, they must be specified by molecules of equal or greater complexity (i.e. other proteins). DNA was known to be a relatively simple molecule, in comparison to proteins, and therefore it was hard to understand how a complex molecule (a protein) could be determined by a simpler molecule (DNA). What were the key experiments which identified DNA as the primary genetic material?

1928 F. Griffith

Background:

Diplococcus pneumoniae, or pneumococcus, is a nasty little bacteria which, when injected into mice, will cause pneumonia and **death** in the mouse. The bacteria contains a **capsular polysaccharide** on its surface which protects the bacteria from host defenses. Occasionally, variants (mutants) of the bacteria arise which have a defect in the production of the capsular polysaccharide. The mutants have two characteristics: 1) They are **avirulent**, meaning that without proper capsular polysaccharide they are unable to mount an infection in the host (they are destroyed by the host defenses), and 2) Due to the lack of capsular polysaccharide the surface of the mutant bacteria appears **rough** under the microscope and can be distinguished from the wild type bacteria (whose surface appears **smooth**).

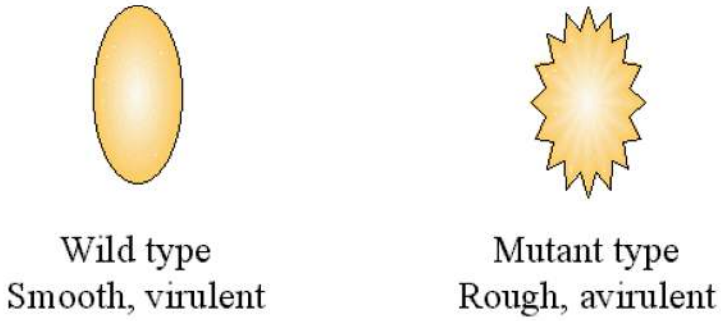


Figure 1.1.1: *Wild type vs. Mutant type pneumococcus*

The virulent smooth wild type pneumococcus can be **heat treated** and rendered **avirulent** (still appears smooth under the microscope however). Finally, there are several different *subtypes* of pneumococcus capsular polysaccharide (subtypes I, II and III). These subtypes are readily distinguishable from one another, and each can give rise to mutants lacking capsular polysaccharide (i.e. the avirulent rough type).

The experiments:

Controls:

- w.t. (smooth) + mouse = dead mouse
- mutant (rough) + mouse = live mouse
- heat treated w.t. (smooth) + mouse = live mouse

Combinations:

- heat treated w.t. (smooth) + mutant (rough) + mouse = **dead mouse**

In this case when the bacteria were recovered from the cold lifeless mouse they were smooth virulent pneumococcus (i.e. indistinguishable from wild type).

A closer look at what is going on, by keeping using, and keeping track of, different *subtypes*

- heat treated w.t. (smooth) type I + mutant(rough) type II + mouse = **dead mouse**

*In this case when the bacteria were isolated from the cold lifeless mouse they were smooth virulent **type I** pneumococcus.*

The overall conclusions from these experiments was that there was a "transforming agent" in the the heat treated type I bacteria which transfomed the live mutant (rough) type II bacteria to be able to produce type I capsule polysaccharide.

Question

Was the "transforming agent" protein or DNA, or what?

1944 O.T. Avery

Background:

The experiment of Griffith could not be taken further until methods were developed to separate and purify DNA and protein cellular components. Avery utilized methods to extract relatively pure DNA from pneumococcus to determine whether it was the "transforming agent" observed in Griffith's experiments.

The experiment:

- w.t. (smooth) type I -> extract the DNA component
- mutant (rough) type II + type I DNA + mouse = dead mouse

Isolation of bacteria from the dead mouse showed that they were type I w.t. (smooth) bacteria

A more sophisticated experiment:

Purified type I DNA was divided into two aliquots. One aliquot was treated with **DNAse** - an enzyme which non-specifically degrades DNA. The other aliquot was treated with **Trypsin** - a protease which (relatively) non-specfically degrades proteins.

- Type I DNA + DNAse + mutant (rough) type II + mouse = live mouse
- Type I DNA + Trypsin + mutant (rough) type II + mouse = **dead mouse**

Conclusion:

The work of Avery provided strong evidence that the "transforming agent" was in fact DNA (and not protein). However, not everyone was convinced. Some people felt that a residual amount of protein might remain in the purified DNA, even after Trypsin treatment, and could be the "transforming agent".

1952 A.D. Hershey and M. Chase

Background:

T2 is a virus which attacks the bacteria *E. coli*. The virus, or *phage*, looks like a tiny lunar landing module:

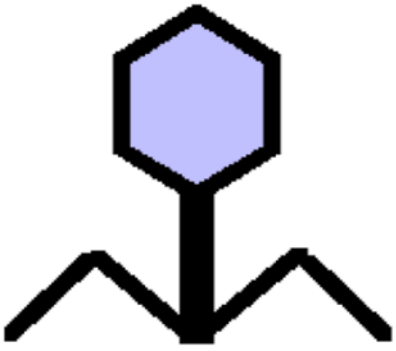


Figure 1.1.2: *T2 phage*

The viral particles adsorb to the surface of the *E. coli* cells. It was known that some material then leaves the phage and enters the cell. The "empty" phage particles on the surface cells can be physically removed by putting the cells into a blender and whipping them up. In any case, some 20 minutes after the phage adsorb to the surface of the bacteria the bacteria bursts open (lysis) and releases a multitude of progeny virus.

If the media in which the bacteria grew (and were infected) included ^{32}P labeled ATP, progeny phage could be recovered with this isotope incorporated into its DNA (normal proteins contain only hydrogen, nitrogen, carbon, oxygen, and sulfur atoms). Likewise, if the media contained ^{35}S labeled methionine the resulting progeny phage could be recovered with this isotope present only in its protein components (normal DNA contains only hydrogen, nitrogen, carbon, oxygen, and phosphorous atoms).

The experiment:

Phage were grown in the presence of either ^{32}P or ^{35}S isotopic labels.

1) *E. coli* were infected with ^{35}S labeled phage. After infection, but prior to cell lysis, the bacteria were whipped up in a blender and the phage particles were separated from the bacterial cells. The isolated bacterial cells were cultured further until lysis occurred. The released progeny phage were isolated.

Where the ^{35}S label went:

- Adsorbed phage shells 85%
- Infected cells (prior to lysis) 15%
- Lysed cell debris 15%
- Progeny phage <1%

2) *E. coli* were infected with ^{32}P labeled phage. The same steps as in 1) above were performed.

Where the ^{32}P label went:

- Adsorbed phage shells 30%
- Infected cells (prior to lysis) 70%
- Lysed cell debris 40%
- Progeny phage 30%

Conclusion:

The material which was being transferred from the phage to the bacteria during infection appeared to be mainly DNA. Although the results were not entirely unambiguous they provided additional support for the view that ***DNA was the "stuff" of genetic inheritance***.

This page titled 10.1.1: DNA as Genetic Material is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **1.1: DNA as Genetic Material** by Michael Blaber has no license indicated.

10.1.2: Structure of DNA and RNA

The Double Helix

DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) are composed of two different classes of nitrogen-containing bases: the **purines** and **pyrimidines**. The most commonly occurring purines in DNA are **adenine** and **guanine**:

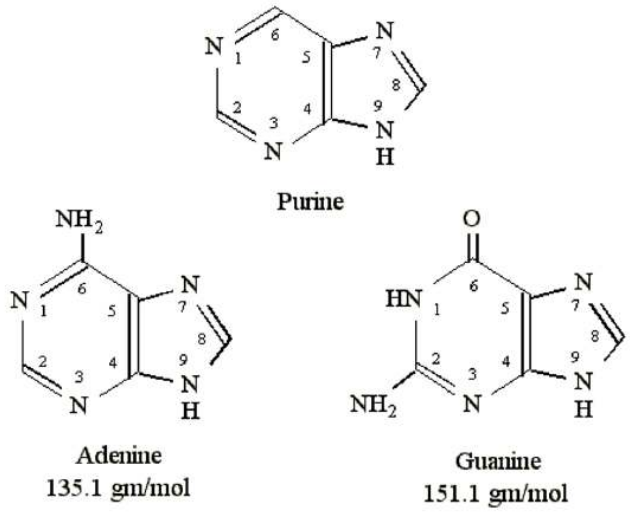


Figure 1.2.1: *Purines*

The most commonly occurring pyrimidines in DNA are **cytosine** and **thymine**:

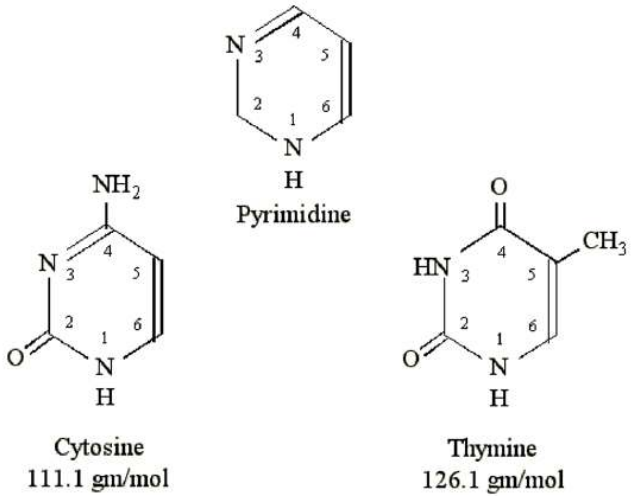


Figure 1.2.2: *Pyramidines*

RNA contains the same bases as DNA with the exception of thymine. Instead, RNA contains the pyrimidine **uracil**:

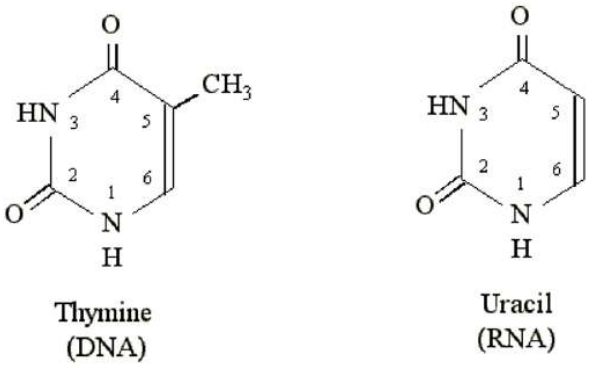


Figure 1.2.3: *Thymine vs. Uracil*

Adenine, guanine, cytosine, thymine and uracil are usually abreviated using the single letter codes A, G, C, T and U, respectively.

Purines and pyrimidines can form chemical linkages with pentose (5-carbon) sugars. The carbon atoms on the sugars are designated 1', 2', 3', 4' and 5'. It is the 1' carbon of the sugar that becomes bonded to the nitrogen atom at **position N1 of a pyrimidine** or **N9 of a purine**. DNA precursors contain the pentose deoxyribose. RNA precursors contain the pentose ribose (which contains an additional OH group at the 2' position):

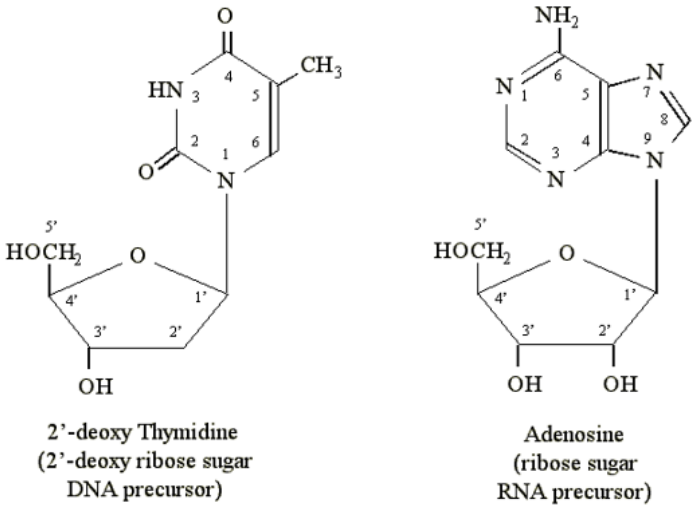


Figure 1.2.4: *Nucleosides*

Before a nucleoside can become part of a DNA or RNA molecule it must become complexed with a phosphate group to form a **nucleotide** (either a deoxyribonucleotide or ribonucleotide). Nucleotides can posess **1, 2 or 3 phosphate groups**, e.g. the nucleotides adenosine monophosphate (AMP), adenoside diphosphate (ADP) and adenosine triphosphate (ATP). The phosphate groups are attached to the **5' carbon** of the ribose sugar moiety. Beginning with the phosphate group attached to the 5' ribose carbon, they are labeled **a, b and g phosphate**. It is the **tri-phosphate nucleotide** which is incorporated into DNA or RNA.

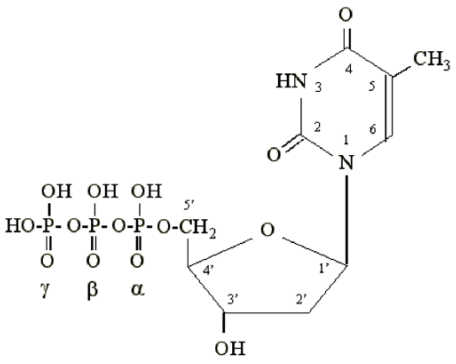


Figure 1.2.5: *Nucleotide*

DNA and RNA are simply long polymers of nucleotides called **polynucleotides**. Only the **a phosphate** is included in the polymer. It becomes chemically bonded to the **3' carbon** of the sugar moiety of another nucleotide:

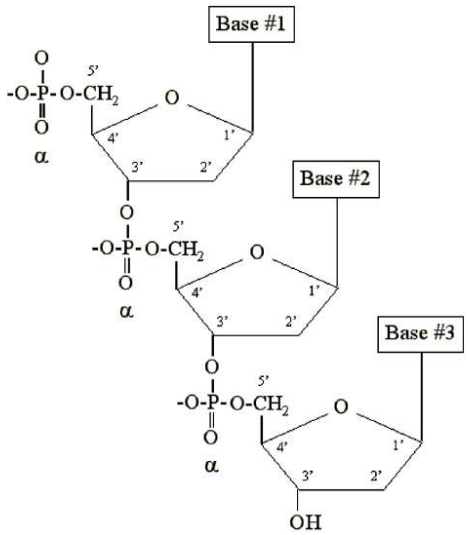


Figure 1.2.6: *Polynucleotide*

In other words, the polynucleotide is connected by a series of 5' to 3' phosphate linkages. Note the sequence of the bases in the above diagram. **Polynucleotide sequences are referenced in the 5' to 3' direction**. Typically, polynucleotides will contain a 5' phosphate and 3' hydroxyl terminal groups. The common representation of polynucleotides is as an **arrow** with the **5' end at the left and the 3' end at the right**.



Summary of terms:

Base	Nucleoside	Nucleotide	RNA (monophosphate)	DNA (monophosphate)	Code
Adenine	Adenosine	(Adenylic acid)	AMP	dAMP	A
Guanine	Guanosine	(Guanylic acid)	GMP	dGMP	G
Cytosine	Cytidine	(Cytidylic acid)	CMP	dCMP	C
Thymine	Thymidine	(Thymidylic acid)		dTMP	T
Uracil	Uridine	(Uridylic acid)	UMP		U

What is the structure of DNA? How is the structure related to function?

1950's

The primary chemical structure of polynucleotides was known (i.e. the 3'-5' phosphate linkage).

1951 E. Chargaff

The experiment:

Take DNA from a variety of species and hydrolyze it to yield individual pyrimidines and purines. Determine the relative concentrations of the A, T, C and G bases.

Result:

Although different species had uniquely different ratios of pyrimidines or purines, the relative concentrations of adenine always equaled that of thymine, and guanine equaled cytosine.

Chargaff's Law: A=T, G=C

1950's R.E. Franklin

X-ray diffraction studies of DNA fibers demonstrated that DNA adopted a highly ordered helical structure. Franklin concluded that two or more chains must coil around each other to form a helix. Some basic dimensions of the helix were calculated from the x-ray diffraction data.

1953 L. Pauling and R.B. Corey

Propose a three chain helical structure for DNA with the phosphate backbone in the center and the bases on the outside.

1953 J.D. Watson and F.H.C. Crick

Identified a hydrogen bonding arrangement between models of thymine and adenine bases, and between cytosine and guanine bases which fullfilled Chargaff's rule:

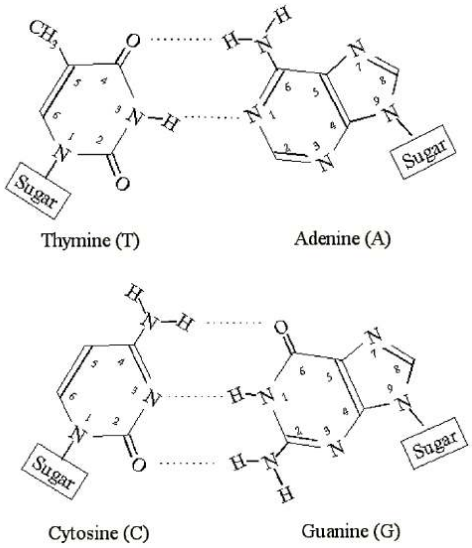


Figure 1.2.7: *Chargaff's Rule Bonding*

Note that the "TA" pair can overlay the "GC" pair with the bonds to the sugar groups in similar juxtaposition. In the "double helix" model of Watson and Crick the polynucleotide chains interact to form a double helix with the chains running in **opposite directions**. The bases are directed towards the center (and stack on top of one another) and the sugar backbones face the outside of the helix.

The Watson and Crick model had the following physical dimensions:

- 34 Å per helical repeat
- 10 base pairs per repeat (i.e. per turn of the helix)
- 3.4 Å inter-base stacking distance
- 20 Å diameter for the helical width

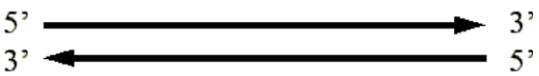
Physical characteristics of the model matched those determined by Rosalind Franklin's x-ray diffraction studies.

Consequenses of the model for genetic information:

The Watson and Crick paper was an exercise in brevity (1 page only in Nature). The structure was so rich with implication that quite a bit could be written. The authors, however, chose only to say "It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material".

1. If G always paired with C, and T always paired with A, then either strand could be regenerated from the **complementary information** in the other strand.
2. The basis of the complementarity was hydrogen bonding, i.e. non-covalent interactions which could be easily broken and re-formed.
3. The information which DNA carried was within the unique **base sequence** of the DNA.
4. From the general interior location of the bases, it would appear that the double helix would **have to dissociate** in order to access the information.
5. The non-equitorial location of the sugar moieties (see above) suggested that the DNA helix would have a **major groove** and a **minor groove**.

General notation of double stranded DNA:



This page titled 10.1.2: Structure of DNA and RNA is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **1.2: Structure of DNA and RNA** by Michael Blaber has no license indicated.

10.1.3: Bacterial Restriction/Modification system

The restriction/modification system in bacteria is a small-scale immune system for protection from infection by foreign DNA.

W. Arber and S. Linn (1969)

Plating efficiencies of bacteriophage lambda (l phage) grown on *E. coli* strains **C**, **K-12** and **B**, when plated on these bacteria:

E. coli strain on which parental phage had been grown	E. coli strain for plating phage		
	C	K-12	B
C	1	$<10^{-4}$	$<10^{-4}$
K-12	1	1	$<10^{-4}$
B	1	$<10^{-4}$	1

- The DNA of phage which had been grown on strains K-12 and B were found to have chemically modified bases which were **methylated**.
- Additional studies with other strains indicate that different strains had specific methylated bases.
- Typical sites of methylation include the **N_6 position of adenine**, the **N_4 position of cytosine**, or the **C_5 position of cytosine**.

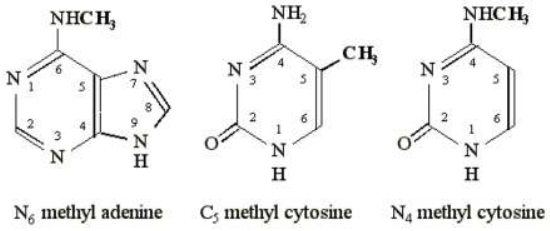


Figure 1.3.1: *Methylation*

- In addition, only a fractional percentage of bases were methylated (i.e. not every adenine was methylated, for example) and these occurred at **very specific sites** in the DNA.
- ***A characteristic feature of the sites of methylation, was that they involved palindromic DNA sequences***.



Figure 1.3.2: EcoR1 methylase specificity. Rubin and Modrich, 1977

- In addition to possessing a particular methylase, individual bacterial strains also contained accompanying specific **endonuclease** activities.
- The endonucleases **cleaved at or near the methylation recognition site**.

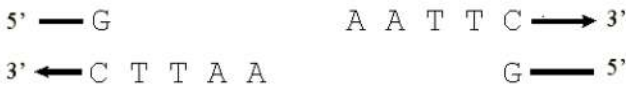


Figure 1.3.3: *Cleavage at methylation sites*

- These specific nucleases, however, would **not** cleave at these specific palindromic sequences if the DNA was **methylated**.

Thus, this combination of a specific methylase and endonuclease functioned as a type of **immune system** for individual bacterial strains, protecting them from infection by foreign DNA (e.g. viruses).

- In the bacterial strain EcoR1, the sequence GAATTC will be methylated at the internal adenine base (by the EcoR1 methylase).
- The EcoR1 endonuclease within the same bacteria will not cleave the methylated DNA.
- Foreign viral DNA, which is not methylated at the sequence "GAATTC" will therefore be recognized as "foreign" DNA and will be cleaved by the EcoR1 endonuclease.
- Cleavage of the viral DNA renders it non-functional.

Such endonucleases are referred to as "restriction endonucleases" *because they restrict the DNA within the cell to being "self"*.

The combination of restriction endonuclease and methylase is termed the "restriction-modification" system.

Of course, this type of protective system is beaten if the attacking phage was previously grown on the same strain as that which it is infecting. In this case the phage will have its DNA already methylated at the appropriate sequence, and will be recognized as "self" (see the table above). *E. coli* strain 'C' (above) is strain which has no known restriction-modification system.

We will discuss DNA replication later, but it should be mentioned that:

- replicating host DNA will initially have one strand (parental) methylated and the other (nascent strand) non-methylated.
- This is recognized as "self" and is not cleaved by the restriction endonuclease.

- It is subsequently methylated by the host methylase.

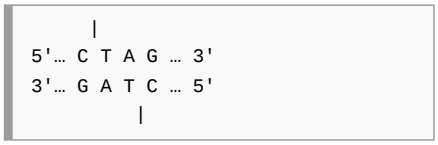
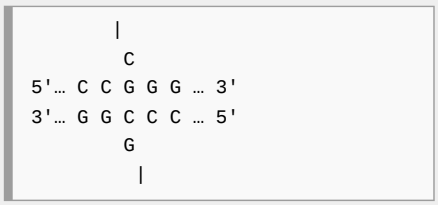
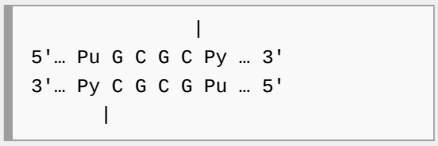
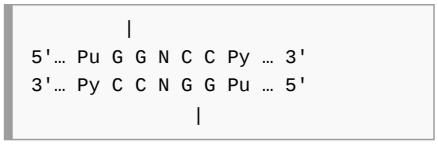
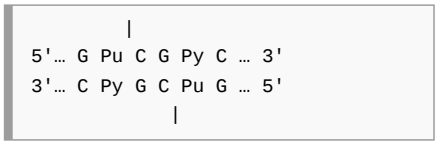
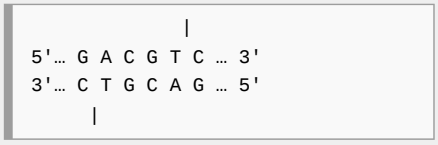
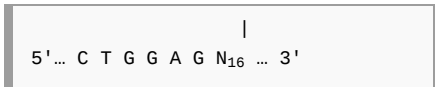
Structural and biochemical studies have indicated that for the common R/M systems (so called ***type II***), the methylase recognizes and methylates one strand of the DNA duplex, whereas the restriction endonuclease recognizes both strands of the DNA (i.e. both strands must be non-methylated for recognition). It is able to do this because it is a homo-dimer protein.

Restriction endonucleases

Since different bacterial strains and species have potentially different R/M systems, their characterization has made available over 200 endonucleases with different sequence specific cleavage sites.

- They are one of the primary tools in modern molecular biology for the manipulation and identification of DNA sequences.
- Restriction endonucleases are commonly named after the bacterium from which it was isolated.

Examples of different restriction enzymes

Name	Source	Recognition Sequence	Comments
Alu I	*Arthrobacter luteus*		"Four cutter". Leaves blunt ends to the DNA.
Bfa I	*Bacteroides fragilis*		"Four cutter". Leaves 5' overhang.
Nci I	*Neisseria cinerea*		"Five cutter". Middle base can be either cytosine or guanine. Leaves 5' overhang. Different recognition sites may have non-complementary sequences.
Eco R1	*Escherichia coli*		"Six cutter". Leaves 5' overhang. Behaves like a "four cutter" ('star' activity) in high salt buffer. $44 for 10,000 units.
Hae II	*Haemophilus aegyptius*		"Six cutter". Pu is any purine, Py is any pyrimidine. Leaves 3' overhang.
EcoO109I	*Escherichia coli*		"Seven cutter". Pu is any purine, Py is any pyrimidine, N is any base. Leaves 5' overhang. Different recognition sites may have non-complementary sequences.
Bgl I	*Bacillus globigii*		"Six cutter with interrupted palindrome". Leaves 5' overhang. Different recognition sites may have non-complementary sequences.
Bsa HI	*Bacillus stearothermophilus*		"Six cutter". Different recognition sites will be complementary.
Aat II	*Acetobacter aceti*		"Six cutter" with 3' overhang. Same recognition sequence as Bsa HI, but different cleavage position.
Bpm I	*Bacillus pumilus*		Non-palindrome, distal cleavage. Leaves 3' overhang. $50 for 50 units.

		3'… G A C C T C N_{14} … 5' |	
Not I	*Nocardia otitidiscaviarum*	| 5'… G C G G C C G C … 3' 3'… C G C C G G C G … 5' |	"Eight cutter". Leaves 5' overhang.
Bsm I	*Bacillus stearothermophilus*	| 5'… G A A T G C N … 3' 3'… C T T A C G N … 5' |	"weird". Leaves 3' overhang.

- The utility of restriction endonucleases lies in their specificity and the frequency with which their recognition sites occur within any given DNA sample.
- ***If there is a 25% probability for a specific base at any given site, then the frequency with which different restriction endonuclease sites will occur can be easily calculated (0.25^n):***

Nucleotide Specificity	Example	Frequency of Occurrence
Four	Alu I	256 (0.25 Kb)
Five	Nci I	1024 (1.0 Kb)
Six	EcoR I	4096 (4.1 Kb)
Seven	EcoO109I	16384 (16.4 Kb)
Eight	Not I	65536 (65.5 Kb)

Thus, on average, any given DNA will contain an Alu I site every 0.25 kilobases, whereas a Not I site occurs once about every 65.5 kilobases.

- Not I is therefore a very useful enzyme for isolating large regions of DNA, typically in research involving genomic DNA manipulations.
- Alu I would be expected to digest a DNA sample into lots of little pieces.

The assortment of DNA fragments would represent a specific "fingerprint" of the particular DNA being digested. Different DNA would not yield the same collection of fragment sizes. Thus, DNA from different sources can be either matched or distinguished based on the assembly of fragments after restriction endonuclease treatment. ***These are termed "Restriction Fragment Length Polymorphisms", or RFLP's***. This simple analysis is used in various aspects of molecular biology as well as a law enforcement and genealogy. For example, genetic variations which distingish individuals also may result in fewer or additional restriction endonuclease recognition sites.

Restriction endonucleases are supplied in various concentrations with activities that are based upon cleavage rates of "standard" DNA samples**.**

- *One unit of activity is typically defined as the amount of enzyme required to digest (or "restrict") one microgram of reference DNA in one hour at 37 °C.*
- The reference DNA may actually have one or more recognition sites for the nuclease in question. DNA's used as "standard" samples may include phage l DNA, or the plasmid pBR322.
- The endonuclease hydrolysis is a spontaneous reaction and does not, for example, require addition of ATP. Reaction buffers for restriction endonucleases usually contain a buffer component (typically 10 mM TRIS buffer around pH 8.0), magnesium salt (often 10 mM $MgCl_2$), a reducing agent (usually 1mM dithiothreitol, or DTT), a protective carrier protein (typically 100 ug/ml bovine serum albumin, or BSA), and salt (sodium chloride).
- ***The biggest determinant of enzyme activity is typically the ionic concentration (NaCl content) of the buffer***. Although there are hundreds of different restriction endonucleases, the majority of them can exhibit between 30-100% activity using a simple system of three buffers, containing either low (20 mM), medium (100 mM) or high (250 mM) salt (NaCl) concentrations in the above described buffer.

  Enzyme digests are typically performed for 1-2 hours at 37 °C. However, quantitative digestion can sometimes only be achieved after extended incubation (i.e. overnight).

This page titled 10.1.3: Bacterial Restriction/Modification system is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **1.3: Bacterial Restriction/Modification system** by Michael Blaber has no license indicated.

10.1.4: DNA Modifying Enzymes

Methylases

Just as the study of the bacterial restriction-modification system has provided a variety of specific endonucleases, there are also available a variety of specific DNA methylases.

- The recognition sequences of the methylases are the same as the associated endonucleases (e.g. EcoR1 methylase recognizes and methylates at the sequence "GAATTC").
- All methylases transfer the methyl group from S-adenosylmethionine (SAM) to a specific base in the recognition sequence, and SAM is a required component in the methylation reaction.
- Methylation of DNA usually has the effect of protecting the DNA from the related restriction endonuclease. However, there are methylases with minimal specificity. For example, Sss I methylase will methylate cytosine residues in the sequence 5' … CG … 3'. In this case, *the methylated DNA will be protected from a wide variety of restriction endonucleases*.
- Some restriction endonucleases will only cut DNA at their recognition sites if the DNA **is** methylated (e.g. Dpn I).
- Still other restriction endonucleases will cut **both** methylated and non-methylated DNA at their recognition sequences (e.g. BamH I).

---

dam and *dcm* Methylation

- The methylase encoded by the *dam* gene (dam methylase) transfers a methyl group from SAM to the N_6 position of the adenine base in the sequence 5' … GATC … 3'.
- The methylase encoded by the *dcm* gene (dcm methylase) methylates the internal cytosine base, at the C_5 position, in the sequences 5' … CCAGG … 3' and 5' … CCTGG … 3'.
- Almost all strains of *E. coli* commonly used in cloning have a *dam+dcm+* genotype. The point here is *not* that we particularly want our DNA to be methylated, but that to make a *dam-dcm-* host someone has to mutate the bacteria and isolate the correct mutant. That apparently has not been done for a lot of bacterial strains. Probably because the *dam* and *dcm* methylation affects only a small subset of potential restriction endonucleases

DNA isolated from *dam+dcm+* strains **will not actually be cut** by a modest subset of available restriction endonucleases:

Recognition sequence	Restriction enzyme	GATC	$G^{me}ATC$
TGATCA	*Bcl I*	+	-
GATC	*Mbo I*	+	-
ATCGAT	*Cla I*	+	-
TCTAGA	*Xba I*	+	-
TCGA	*Taq I*	+	-
GAAGA	*Mbo II*	+	-
GGTGA	*Hph I*	+	-

DNA may have to be prepared from E. coli strains which are dam-dcm- in order to be cut by these enzymes.

DNA Polymerases

A wide variety of polymerases have been characterized and are commercially available. All DNA polymerases share two general characteristics:

1. They add nucleotides to the 3'-OH end of a primer

2. The order of the nucleotides in the nascent polynucleotide is template directed

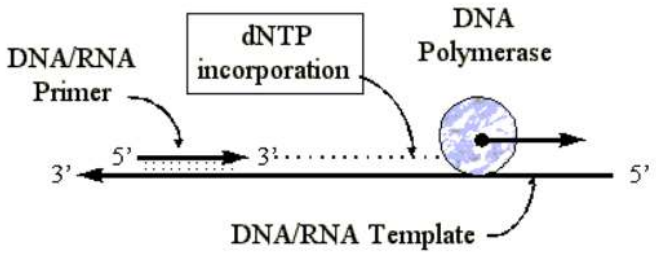


Figure 1.4.1: *DNA Replication*

In addition to the 5'->3' polymerase activity, polymerases can contain exonuclease activity. This exonuclease activity can proceed either in the 5'->3'direction, or in the 3'->5' direction.

- Exonuclease activity in the 3'->5' direction allows the polymerase to correct a mistake if it incorporates an incorrect nucleotide (so called "***error correction activity***"). *It can also slowly degrade the 3' end of the primer*.
- Exonuclease activity in the 5'->3' direction will allow it to degrade any other hybridized primer it may encounter. Without 5'->3' exonuclease activity, obstructing primers may or may not be physically deplaced, depending on the polymerase being used.

Different polymerases have differing error rates of misincorporation, and different rates of polymerization.

E. coli DNA polymerase I	E. coli DNA polymerase I - Klenow Fragment	T4 DNA polymerase	T7 DNA polymerase	Taq DNA polymerase	M-MuLV Reverse Transcriptase
5'->3' exonuclease activity	*				*
3'->5' exonuclease activity	*	*	*	*	
Error Rate ($x10^{-6}$)	9	40	<1	15	285
Strand Displacement		*			
Heat Inactivation	*	*	*	*	

Uses of polymerases

The various activities of the different polymerases lend them to a variety of applications. For example, restriction endonucleases can yield fragments of DNA with either 3' or 5' nucleotide "overhangs".

- In the case of 5' overhangs, the 5'->3' polymerase activity can fill these in to make **blunt ends**.
- In the case of 3' overhangs, the 3'->5' exonuclease activity present in some polymerases (especially T4 DNA polymerase) can "chew back" these ends to also make blunt-ended DNA fragments.

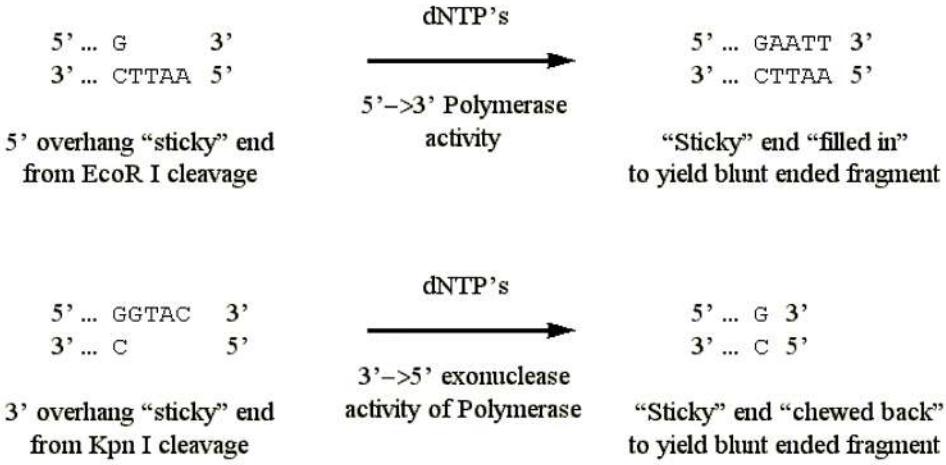


Figure 1.4.2: *Polymerase activity*

"Nick-translation"

This method is used to obtain highly radiolabeled single strand DNA fragments, which makes use of 5'->3' exonuclease activity present in some polymerases (*E. coli* DNA polymerase I, for example).

- In this method a DNA duplex of interest is "nicked" (i.e. one of the strands is cut; see DNAse I).
- Then DNA pol I is added along with radiolabeled nucleotides. The 5'->3' exonuclease activity chews away the 5' end at the "nick" site and the polymerase activity incorporates the radiolabeled nucleotides. The resulting polynucleotide will be highly radiolabeled and will hybridize to the DNA sequence of interest.

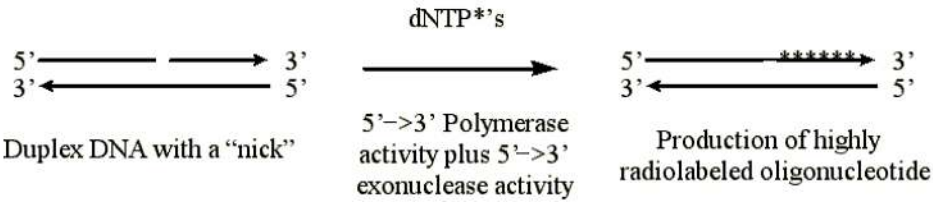


Figure 1.4.3: *Nick-translation*

- Thermostable polymerases have the ability to remain functional at temperature ranges where the DNA duplex will actually "melt" and become separated. This has allowed the development of the **"Polymerase Chain Reaction"** technique (PCR), which has had a profound impact on modern Biotechnology. We will discuss this method at a later date.
- The incorporation of dideoxy bases (i.e. no hydroxyl groups on either the 2' or 3' carbon of the ribose sugar) leads to **termination of the polymerase reaction**. This will be discussed in greater detail later. However, this chain termination by incorporation of dideoxynucleotides is the basis of the Sanger method of DNA sequencing, as well as therapies to try to inhibit viral replication.

Nucleases

Nuclease BAL-31

- This is an exonuclease (starts at the termini and works inward) which will degrade both 3' and 5' termini of double-stranded DNA. It will not make internal cleavages ("nicks"), however, it will degrade the ends of DNA at existing internal "nicks" (which create both 3' and 5' termini).
- The degradation of termini is not coordinated, meaning that the product is not 100% blunt-ended (even though the original duplex may have been blunt ended).
- Such "ragged" ends can be made blunt by filling in and chewing back by a suitable polymerase (e.g. T4 DNA polymerase). The unit definition of 1 unit is the amount of enzyme required to remove 200 base pairs from each end of duplex DNA in 10 minutes

at 30 °C.

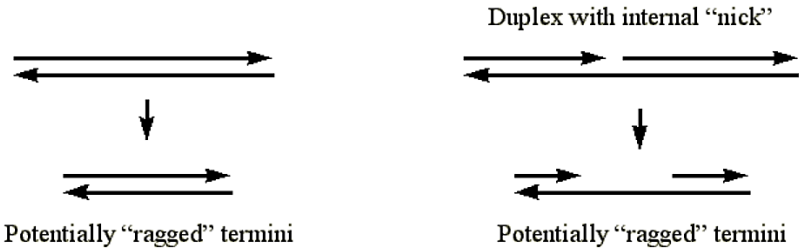


Figure 1.4.4: *Nuclease BAL-31 activity*

Exonuclease III

- Catalyzes the stepwise removal of nucleotides from the 3' hydroxyl termini of duplex DNA.
- The enzyme will attack the 3' hydroxyl at duplex DNA with blunt ends, with 5' overhangs, or with internal "nicks".
- Since duplex DNA is required, the enzyme **will not** digest the 3' end of duplex DNA where the termini are 3' overhangs.

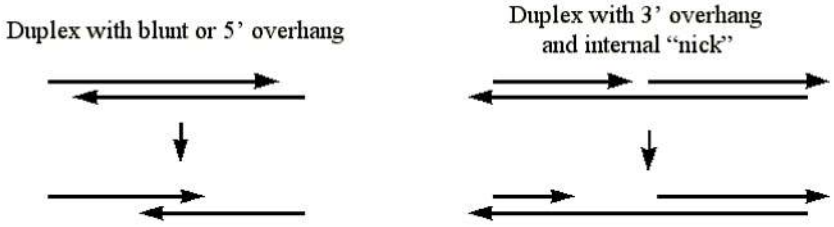


Figure 1.4.5: *Exonuclease III Activity*

Mung Bean Nuclease (isolated from mung bean sprouts)

- A single strand specific DNA and RNA endonuclease which will degrade single strand extensions from the ends of DNA and RNA leaving blunt ends.
- The single strand extensions can be either 5' or 3' extensions - both are removed and a blunt duplex is left.

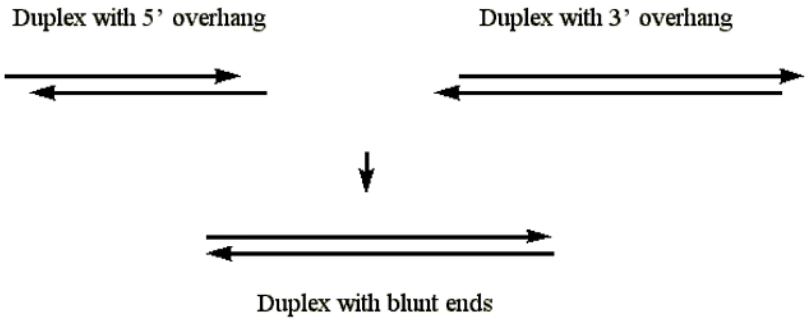


Figure 1.4.6: *Mung Bean Nuclease activity*

Deoxyribonuclease I (DNAse I) from Bovine pancrease

- This enzyme hydrolyzes duplex or single DNA strands preferentially at the phosphodiester bonds 5' to pyrimidine nucleotides
- In the presence of Mg^{2+} ion, DNAse I attacks each strand independently and produces nicks in a random fashion (useful for nick-translation)
- In the presence of Mn2+ ion DNAse I cleaves both strands of DNA at approximately the same position (but leaving "ragged" ends)

Ligases

- Ligases catalyze the **formation** of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini of nucleotides (potentially RNA or DNA depending on the ligase).
- In a sense, they are the opposite of restriction endonucleases, but they do not appear to be influenced by the local sequence, *per se*.
- Ligases require **either rATP or NAD+ as a cofactor**, and this contrasts with restriction endonucleases.

The following are different types of ligases and their characteristics.

T4 DNA ligase

- Isolated from bacteriophage T4.
- Will ligate the ends of duplex DNA or RNA.
- This enzyme will join blunt-end termini as well as ends with cohesive (complementary) overhanging ends (either 3' or 5' complementary overhangs).
- This enzyme will also repair single stranded nicks in duplex DNA, RNA or DNA/RNA duplexes. Requires **ATP** as a cofactor.

Taq DNA ligase

- This ligase will catalyze a phosphodiester bond between two adjacent oligonucleotides which are hybridized to a complementary DNA strand:

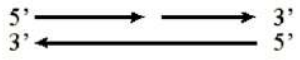


Figure 1.4.7: *Taq DNA ligase activity*

- The ligation is efficient only if the oligonucleotides hybridize perfectly with the template strand.
- The enzyme is active at relatively high temperatures (45 - 65 °C). Requires **NAD+** as a cofactor.

T4 RNA ligase

- Will catalyze formation of a phosphodiester bond between RNA/RNA oligonucleotides, RNA/DNA oligonucleotides, or DNA/DNA oligonucleotides.
- Requires **ATP** as a cofactor.
- This enzyme does not require a template strand.

T4 RNA ligase can be used for a variety of purposes including constructing RNA/DNA hybrid molecules.

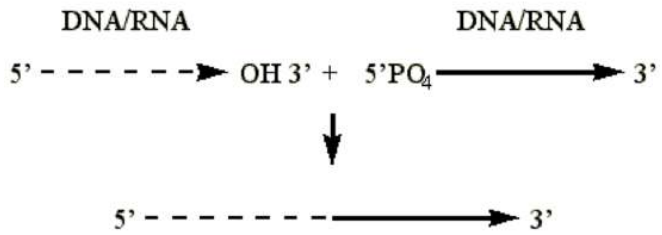


Figure 1.4.8: *T4 RNA ligase activity*

DNA ligase (E. coli)

- Will catalyze a phosphodiester bond between duplex DNA **containing cohesive ends**.
- It will **not** efficiently ligate blund ended fragments.
- Requires **NAD+** as a cofactor.

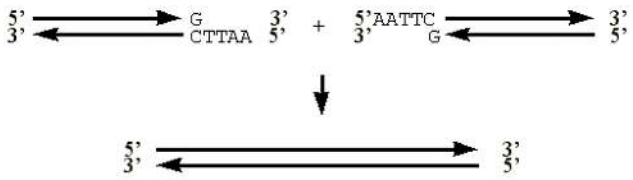


Figure 1.4.9: *DNA ligase (E. Coli) activity*

T4 polynucleotide kinase

- Catalyzes the transfer and exchange of a phosphate group from the **g** position of rATP (adenine ribose triphosphate nucleotide) to the 5' hydroxyl terminus of double stranded and single stranded DNA or RNA, **and** nucleoside 3' monophosphates.
- The enzyme will also remove 3' phosphoryl groups.
- **Oligonucleotides which are obtained from automated synthesizers lack a 5' phosphate group, and thus, cannot be ligated to other polynucleotides**.

T4 polynucleotide kinase can be used to phosphorylate the 5' end of such polynucleotides:

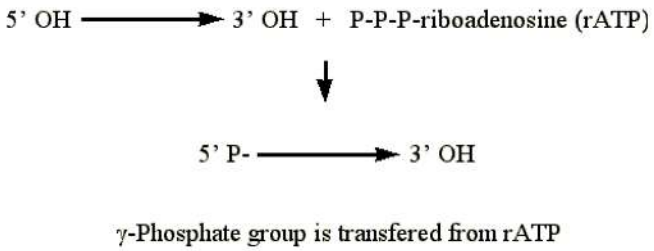


Figure 1.4.10: *T4 polynucleotide kinase activity*

Calf intestinal phosphatase (CIP)

- Catalyzes the removal of 5' phosphate groups from RNA, DNA and ribo- and deoxyribo- nucleoside triphosphates (e.g. ATP, rATP).
- CIP treated duplex **DNA cannot self ligate**.
- Hemi-phosphorylated duplexes will be ligated on one strand (the phosphorylated strand) and remain "nicked" on the other.

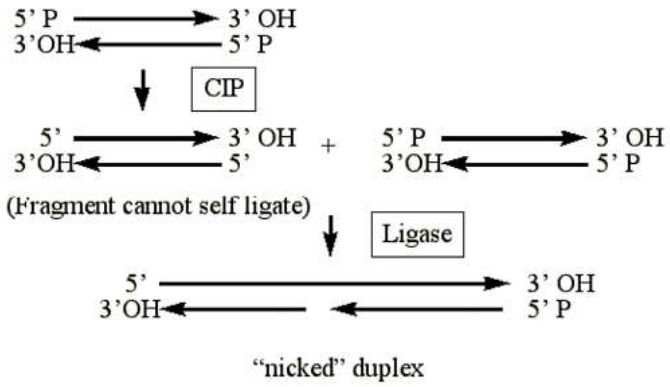


Figure 1.4.11: *CIP activity*

This page titled 10.1.4: DNA Modifying Enzymes is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **1.4: DNA Modifying Enzymes** by Michael Blaber has no license indicated.

10.1.5: DNA Replication - Introduction to Prokaryotic replication

1953

- The DNA model of Watson and Crick suggested how genetic information might be replicated: either strand of the duplex can be used as a template to replicate the sequence information.
- But, was the replication conservative (i.e. the original parental strands remain together after replication) or semi-conservative(one parental strand pairs with one newly synthesized strand)?

The answer for prokaryotic organisms (i.e. lack a true membrane bound nucleus and cellular organelles; e.g. bacteria) came from the 1958 experiment of Meselson and Stahl.

1958

The nitrogen in ammonium salts in culture broth is incorporated into DNA bases. The most common isotope of nitrogen is ^{14}N. However, ^{15}N ammonium salts (a heavier isotope) can also be obtained.

- DNA from *E. coli* cells grown with ^{15}N ammonium salts will have a *higher density* than DNA grown in "normal" (^{14}N) ammonium salts.
- Such DNA will migrate differently on cesium chloride ($CsCl_2$) equilibrium density gradient centrifugation.
- The more dense DNA will migrate as a lower band (on this type of centrifugation the characteristic migration position is a function of density, and *is independent of DNA length*).

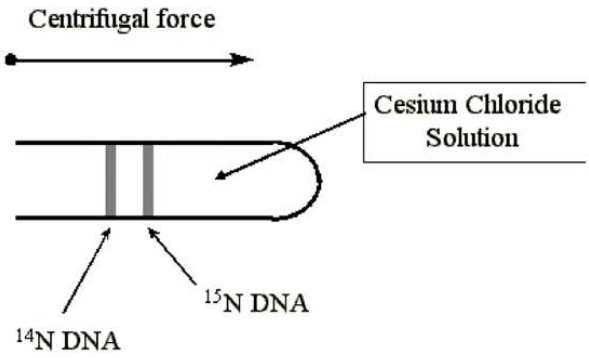


Figure 1.5.1: *Density of ^{15}N and ^{14}N* DNA

Meselson and Stahl reasoned that if they grew *E. coli* in ^{15}N salts then ***switched media to ^{14}N salts*** for additional rounds of replication, the mode of replication could be deduced from the density of the DNA.

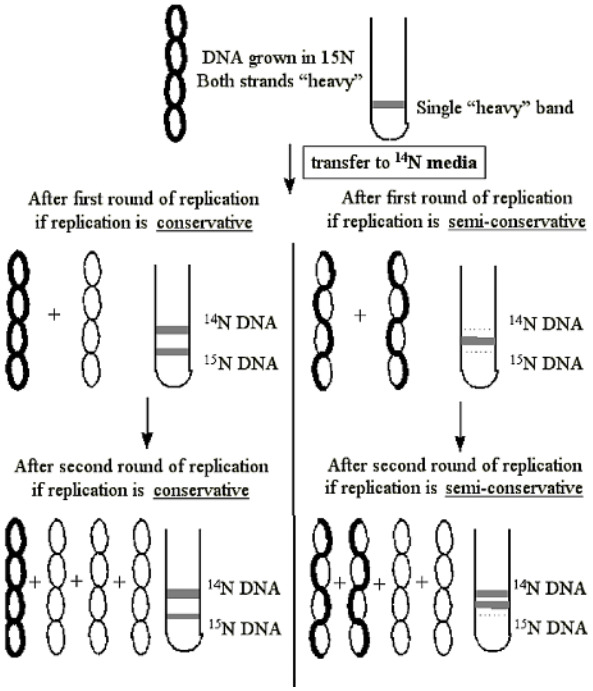


Figure 1.5.2: *Meselson and Stahl experiment*

- After switching to the ^{14}N media and allowing the cells to go through a round of replication a single band of intermediate density was observed (i.e. between ^{14}N and ^{15}N control DNA samples).
- After a second round of replication in ^{14}N media two bands were present in approximately equimolar amounts; one was intermediate in density and the other migrated as purely ^{14}N labeled DNA.

The results were consistent with a semi-conservative mode of replication for DNA. Evidence of semi-conservative replication of DNA has since been obtained with both plant and animal DNA.

DNA Replication in *E. coli*

E. coli DNA polymerase characteristics:

- Polymerase will only elongate an existing polynucleotide. It ***cannot initiate polynucleotide formation***:

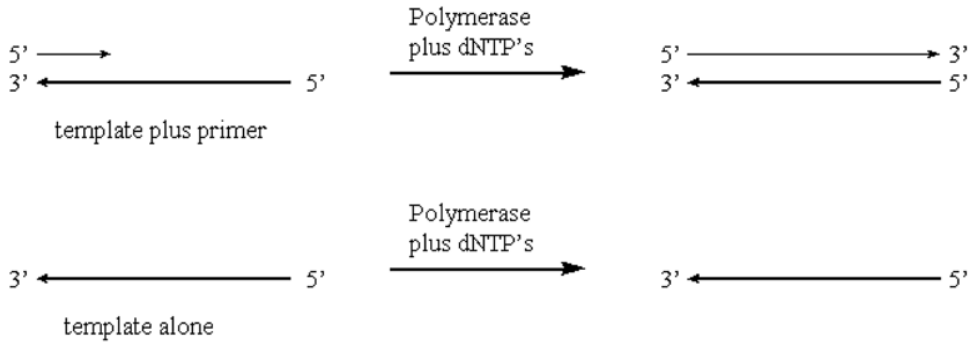


Figure 1.5.3: *DNA Polymerase activity*

- Polymerase will catalyze polymerization of nucleotides only in one direction (5'>3') via a phosphodiester bond between a 3' hydroxyl and 5' phosphate group.
- DNA polymerase is unable to unwind duplex DNA to separate the two strands which need to be copied

E. coli genome is circular duplex DNA of approximately 4 x 10^6 base pairs (i.e. 4 Mb)

- The genome has a single origin of replication.
- ***DNA duplication in E. coli begins at a specific site in the DNA called "oriC"***.

OriC is a region of DNA approximately 240 nucleotides long.

- It contains repetitive 9-base pair and 13-base pair sequences (known as the '9-mer' and '13-mer' regions).
- These sequences are AT rich regions, which *melt at lower temperatures* than DNA containing GC pairs.
- These regions are postulated to help melt the DNA duplex in the oriC region for initiation of DNA replication.

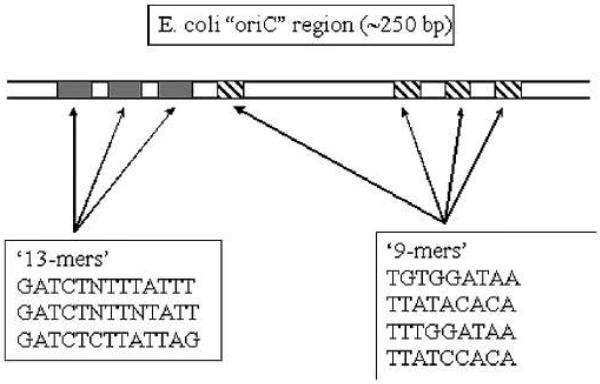


Figure 1.5.4: *oriC region*

The **dnaA** gene product: (dnaA protein)

- Strains of *E. coli* with mutations in the dnaA gene were able to grow at 30 °C, but not at 39-42 °C.
- However, if DNA synthesis was begun at 30 °C, and then the temperature was shifted to 42 °C, DNA synthesis continued until the genome was replicated (and the cell divided), but no new initiationof DNA synthesis was possible.

Conclusion: *Somehow the product of the dnaA gene (i.e. the dnaA protein) is required for initiation of DNA synthesis*.

Studies of purified dnaA protein:

- dnaA protein binds to the '9-mer' region in oriC and forming a multimeric complex with 10-20 protein subunits (i.e. at a single oriC region there will be bound 10-20 dnaA protein molecules).
- Binding requires ATP.
- Further addition of ATP was observed to result in a *melting and opening up* of the DNA duplex in the oriC region. This was determined by addition of *S1 nuclease* (like mung bean, but will also cut DNA at the site of an internal nick), which resulted in cleavage of DNA at the site of oriC.

The **dnaB** gene product: (dnaB protein)

The protein encoded by the dnaB gene appears to be essential for DNA replication. The dnaB protein has been identified as ahelicase. A helicase moves along a DNA strand opening up the duplex to melt and separate the DNA strands.

- dnaB protein binds to the single stranded DNA in the general region of the oriC DNA segment.
- Binding requires ATP as well as the dnaC gene product (the dnaC protein).
- After helicase/dnaC binds to the DNA, the dnaC protein is released.
- Two helicases bind at the oriC region, one helicase on each strand of the DNA.

This stage represents the prepriming complex:

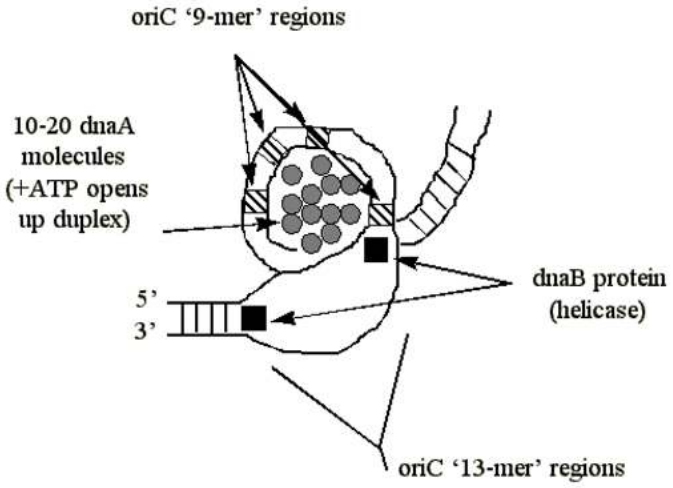


Figure 1.5.5: *Prepriming complex*

Separated strands in the oriC region are prevented from reannealing by the binding of *single-stranded binding protein* (*ssb* protein).

The dnaG gene protein:

The dnaG gene protein is called ***primase***.

- Primase catalyzes synthesis of short RNA molecules that function as primers for DNA synthesis by *E. coli* DNA polymerase III(pol III).
- Primase binds to dnaB protein at oriC and forms a ***primosome***.
- The primase within the primosome complex provides RNA primers for synthesis of both strands of duplex DNA.
- **Primase lays down tracks of pppAC(N)$_{7\text{-}10}$** (RNA).

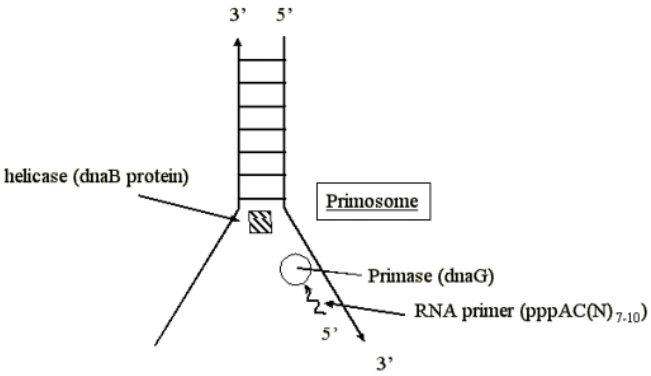


Figure 1.5.6: *Primase activity*

- After synthesis of the 9-12 mer RNA primer, DNA Pol III holoenzyme enters the replication fork and is able to utilize the RNA as a primer for DNA synthesis.
- As the replication fork opens up, the leading strand synthesis can continue, but ***a gap develops in the lagging strand***:

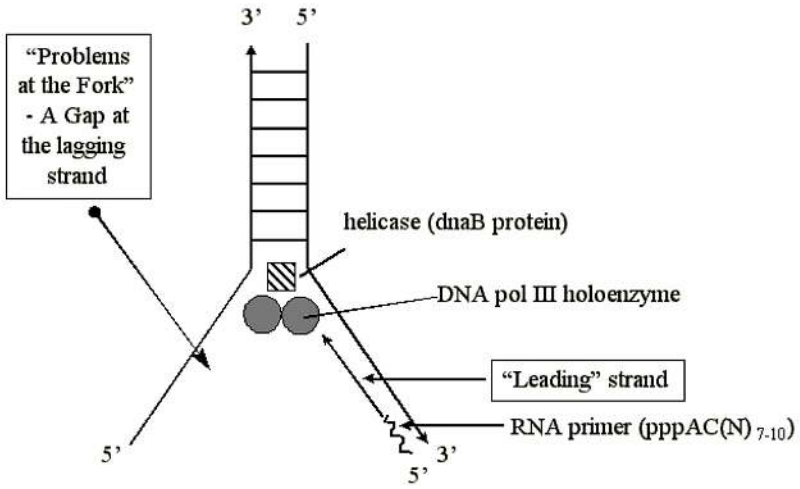


Figure 1.5.7: *Lagging Strand*

DNA Pol III is a large multicomplex enzyme (holoenzyme) which is somewhat dimeric in nature (there are two polymerase active sites). The two active polymerase sites in Pol III could actually function to synthesize both nacent strands at the fork. However, the synthesis of the lagging template strand would be in the opposited direction to the movement of the Pol III complex:

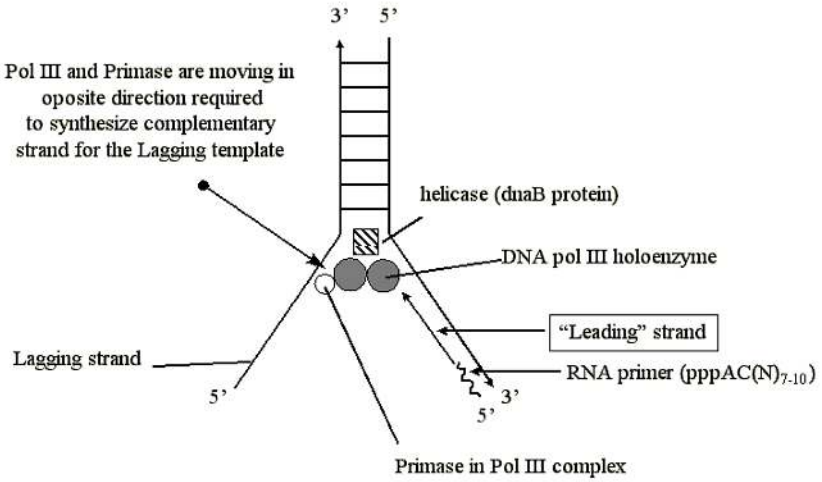


Figure 1.5.8: *Pol III movement*

Primase can bind to the Pol III complex, but the arrangement of the DNA strand as it passes through the Pol III/primase complex is quite unique. ***It forms a loop structure such that primase and the Pol III active site can accomplish discontinuous synthesis of the lagging template strand even though the general direction of the Pol III complex is opposite to the require direction of DNA synthesis***:

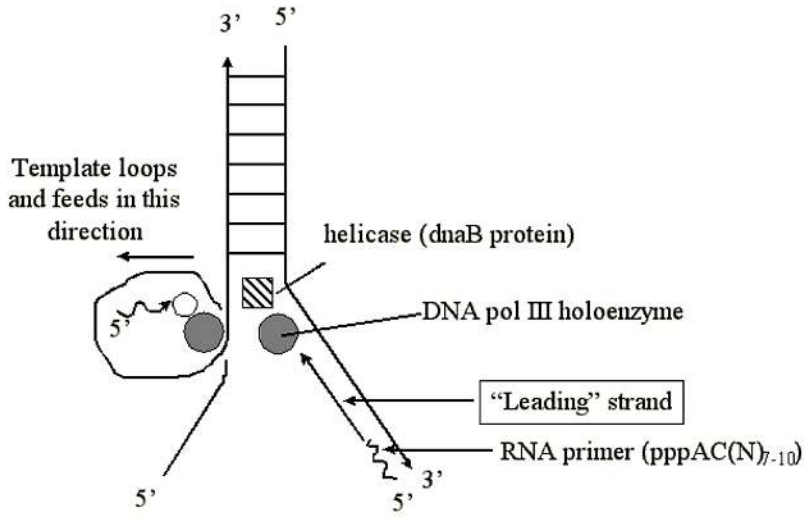


Figure 1.5.9: *Loop for Pol III activity*

After primase makes another primer on the lagging template, the adjacent Pol III active site can extend the primer (incorporating dNTP's) by utilizing the same loop structure and feeding the template through in the direction shown.

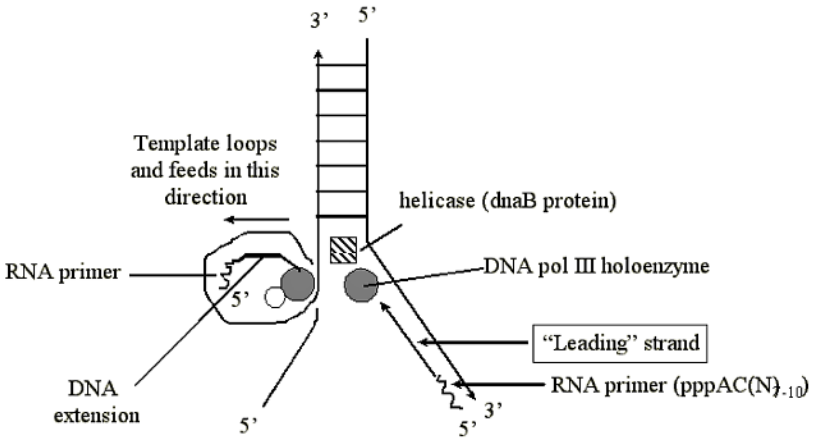


Figure 1.5.10: *Synthesis of lagging strand*

The lagging strand loop cannot be fed through the Pol III complex forever, and after a nascent DNA strand is synthesized the loop is released and a new one is formed using the opened template DNA further up the fork:

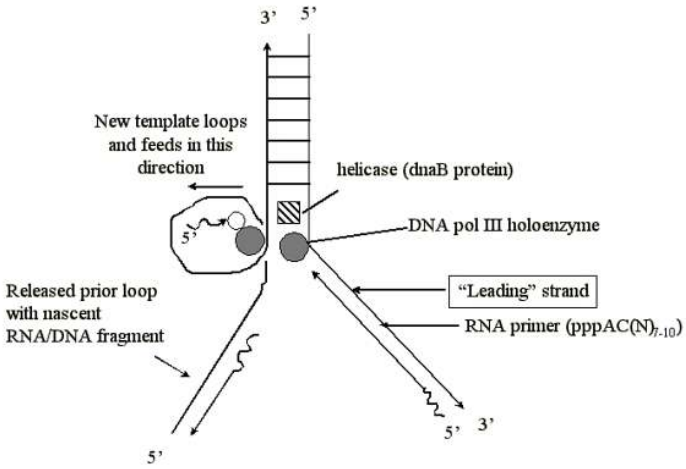


Figure 1.5.11: *Continuation of lagging strand synthesis*

As synthesis continues:

- there will be a single *continuous DNA strand* on the *leading strand*
- there will be a series of *short fragments on the lagging strand*, containing both RNA and DNA, called ***Okazaki fragments***:

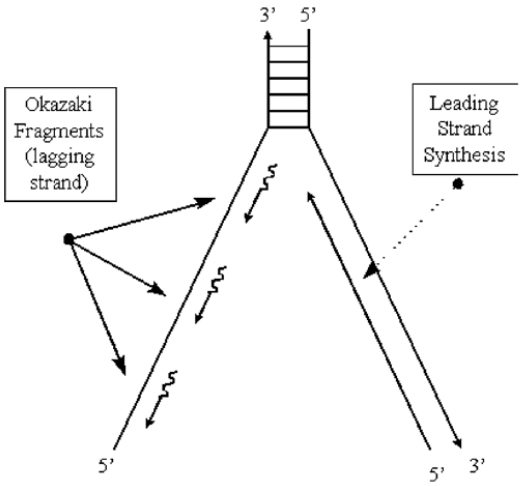


Figure 1.5.12: *Okazaki Fragments*

How are these RNA/DNA fragments converted into one long continuous DNA strand? The RNA could be removed by a polymerase which has 5'->3' exonuclease activity, however, *Pol III lacks this activity*.

- *DNA Pol I* does have 5'->3' exonuclease activity
- it can extend the DNA synthesis via nick-translation.
- The nick-translation activity restults in degradation of the RNA primers.
- The end result is a series of "nicks" in the lagging strand, now *100% DNA*:

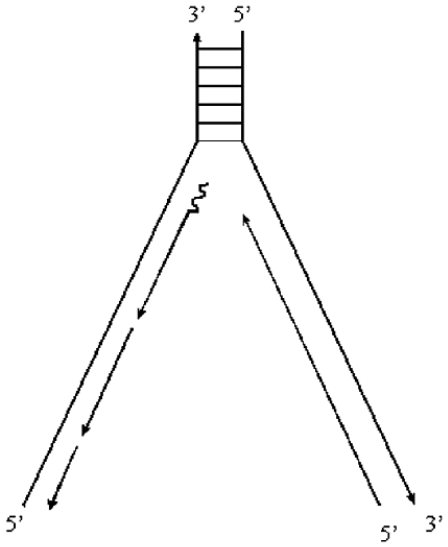


Figure 1.5.13: *Nicks in lagging strand*

- DNA Pol I leaves and DNA ligase then joins these discontinuous DNA fragments to form a continuous DNA duplex on the lagging strand.

Summary of steps in *E. coli* DNA Synthesis

1. dnaA protein melts duplex in oriC region.
2. dnaB (helicase), along with dnaC and ATP binds to replication fork (dnaC protein exits).[1] (**Pre-priming complex**)
3. Single strand binding protein (ssb protein) binds to separated strands of DNA and prevents reannealing.
4. Primase complexes with helicase, creates RNA primers ($pppAC(N)_{7\text{-}10}$) on the strands of the open duplex[2] (Primase+helicase constitute the **Primosome**).
5. After making the RNA primers, **DNA pol III holoenzyme comes in** and extends the RNA primer (laying down dNTP's) on the leading strand.
6. As the replication fork opens up (via helicase + ATP action) leading strand synthesis is an uninterrupted process, the lagging strand experiences a **gap**.
7. The gap region of the lagging strand can wind around one active site unit of the Pol III complex, and bound Primase **initiates an RNA primer in the gap region[3].**
8. On the lagging strand, Pol III extends the RNA primer with dNTP's as the lagging template strand is looped through the Pol III complex
9. After synthesis of a nascent fragment the lagging strand loop is released and the single strand region further up near the replication fork is subsequently looped through the Pol III complex.
10. Steps 7-9 are repeated.
11. Meanwhile, Pol I removes the RNA primer regions of the Okazaki fragments via 5' to 3' exonuclease activity (nick translation
12. Pol I exits and ligase joints the DNA fragments (on lagging strand).

Notes From Above

1. Polymerases are unable to open up duplex DNA, thus the requirement for helicase
2. Polymerases cannot replicate a DNA template in the absence of a primer (either DNA or RNA).
3. Polymerases extend a polynucleotide in the 5' to 3' direction only. Gaps at the 5' end must be filled by "upstream" discontinuous synthesis.

Properties of E. coli polymerases (Pol I, II and III)

	DNA Pol I	DNA Pol II	DNA Pol III
5'->3' Polymerase activity	*	*	*
3'->5' Exonuclease activity(proof reading)	*	*	*
5'->3' Exonuclease activity(nick translation)	*		
Synthesis from:			
Duplex DNA			
Primed single strand	*		
Primed single strand plus ssb protein	*		*
Chain elongation rate(*in vitro*) bp/min	600	?	30,000
Molecules/cell	400	?	10-20
Mutation Lethal?	*		*

Pol Functions:

- Pol I: gap filling during DNA synthesis and repair, removal of RNA primers
- Pol II: involved in DNA synthesis of damaged templates
- Pol III: functional polymerase at the replication fork

Pol III Structure and function

- A "holoenzyme" complex of 10 different polypeptides
- resultant molecular weight is greater than 600 KDa (i.e. it is a large complex).
- It is structurally an asymmetric dimer - it contains two copies of most of the polypeptides which comprise it, including two catalytic sites for nucleotide addition (i.e. polymerization).

The various protein subunits have a variety of functions:

1. Subunits for polymerase activity: a, e, subunits
2. Subunits to dimerize the core polymerase (t)
3. Subunits to increase processivity (i.e. to increase the ability to synthesize long stretches w/o releasing from the DNA template): b subunits
4. Subunits to bind b to DNA-primer substrate: (g, d, d', c,)

Termination of DNA replication

- Specific termination sites of DNA replication exist in *E. coli*.
- Termination involves the binding of the *tus* gene product (*tus* protein).
- This protein may act to prevent helicase from unwinding DNA (will therefore halt *pol III* and *pol I* action).
- DNA replication produces two interlocking rings which must be separated.
- This is accomplished via the enzyme *topoisomerase*.

ColE1 Plasmid

E. coli can contain a small extrachromosomal element called the ColE1 plasmid. This plasmid has the following general features:

- 6.4 Kb circular duplex DNA
- Autonomously replicating

- 10-15 copies per cell (i.e. per *E. coli* chromosome)

Although it is autonomously replicating, it does not contain an *oriC* type of sequence for initiation of replication, and does not undergo the same steps in replication.

- The plasmid produces (among other things) two RNA oligonucleotides (RNA I and RNA II)
- RNA II has complementarity to the ColE1 origin of replication, which contains an AT rich sequence
- The bound RNA II molecule can serve as a primer for polIII
- Since RNA II binds on one strand only, the replication of the ColE1 plasmid is ***unidirectional***
- RNA I has complementarity with RNAII, and such a duplex RNA cannot serve as a replication primer, thus control of the plasmid ***copy number*** is achieved by the interaction between RNA I and RNA II.

This page titled 10.1.5: DNA Replication - Introduction to Prokaryotic replication is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **1.5: DNA Replication - Introduction to Prokaryotic replication** by Michael Blaber has no license indicated.

10.1.6: DNA Supercoiling and Topoisomerases

Unwinding of the helix during DNA replication (by the action of helicase) results in supercoiling of the DNA ahead of the replication fork.

- This supercoiling increases with the progression of the replication fork.
- If the supercoiling is not relieved, it will physically prevent the movement of helicase.

The topology of DNA can be described by **three** parameters:

1. **Linking Number (L) An integer value. "Positive" is referenced as right-handed.**
2. **Twist (T) A real number (the "apparent" linkage number)**
3. **Writhe (W) A real number ("supercoils" in the DNA structure)**

Consider closed circular DNA:

- **Linking number** is an integer value.
- It refers to the number of times the two strands of the duplex make a complete 360 degree turn.

For circularly closed DNA, like the *E. coli* genome, the linking number can only be changed if we do the following:

1. physically break the duplex
2. introduce (or remove) a 360 degree turn
3. ligate (covalently close) the break.

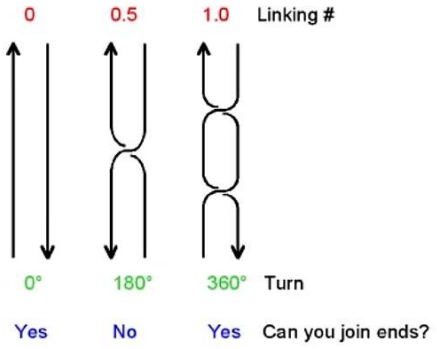


Figure 1.6.1: *Turns to change the linking number*

Rubber tubing "helix" experiment

Cut two lengths of 1/8" rubber tubing, each about 20" long. Insert a smaller piece of tubing, or piece of pipette tip in the ends to allow the ends to be connected. These two pieces of rubber tubing represent each strand of a DNA duplex. DNA can be ligated, or joined, when we have 5' (phosphate) and 3' (hydroxyl) ends. So we need a way to keep track of which end is which for each piece of tubing.

1. You can either write "5" and "3" on opposite ends of each piece and align them in opposite directions, or
2. On one piece of tubing, mark both ends with a sharpie. Only similarly colored ends can be ligated (see diagram above)

This will allow you to maintain correct strand "orientation" when you "ligate" the strands of the duplex.

- Introduce a Linking number = +2 (two 360° right handed twists into the duplex)
- then "ligate" the ends (make sure you maintain strand orientation, i.e. you connect the appropriate two strands).

Confirm that the correct linking number has been introduced by "melting" the duplex on one side and forcing all turns into a small region of the duplex (easy to count this way).

- Confirm that looking down the helix at the turns that they are **"right handed"** (does not matter which way you look down the helix).

Note that the "duplex" when held between thumb and forefinger and allowed to hang, prefers a "supercoiled" topology, as opposed to "relaxed" [Note: this is usually seen with very skinny tubing, larger diameter tubing may not readily adopt this topology].

- Confirm that the "supercoils" are actually ***left handed*** as you look down the supercoils (regardless of direction down the supercoils).
- The "supercoil" is most likely a full 360°, rather than 180°. In any case, hold the ends of the duplex so that a left handed 360° "supercoil" is present.
- Now, count how many times the strands of the "duplex" cross each other. In this conformation, the strands of the "duplex" will ***not*** actually cross each other (Note: you may have one strand crossing and then later uncrossing, for a net result of no crossing).

Thus, in response to the introduction of +2 Linking number, the "duplex" can adopt +2 (180°) "supercoils", such that the resulting **apparent linkage number** (i.e. "**twist**" value) of the two strands is zero

> **Note**
>
> A supercoil is considered positive, if it is "left handed").

- Remove one "positive" supercoil by unwinding by 180°.
- Now hold the ends of the "duplex" and count the **apparent linkage number** (i.e. "**twists**"). There will be a single right-handed "**twist**".
- **Thus, a single 180° "positive" supercoil has the effect of removing a single "positive" twist (i.e. reduces the apparent linkage number by 1).**

*The **Writhe** number refers to the number of supercoils present.*

- Although it may seem that the consequence of introducing ***supercoiling*** (Writhe) is changing the Linking number, **it is not**.
- The consequence of Writhe is that the **Twist (*apparent linkage number*)** is altered (increased or decreased).

DNA has a preferred **"Twist" value** (preferred apparent linkage number) **for a specified length** of DNA:

- Watson and Crick's model of the DNA duplex had 10 basepairs per turn.
- Under physiological conditions of salt (0.15 M NaCl) and temperature, ***DNA prefers to adopt about 10.6 bp/turn***.
- **Writhe is introduced in the DNA to achieve this value for the "Twist" (apparent linkage number)**

For a given (fixed) Linkage number over a given length of DNA, the DNA can adopt either positive or negative supercoils to achieve a "twist" (apparent linkage number) such that there will be 10.6 basepairs/turn.

Linkage number does not change with supercoiling (it can only change by breaking the duplex)

- Writhe has the effect of changing the apparent Linkage number.
- One supercoil is defined as being able to change the apparent linkage number by +/- 1.

The twist value (apparent linkage number) for a given length of DNA is related to the number of base pairs per turn that the DNA wants to adopt:

Linkage Number = (size of DNA in base pairs)/(basepairs/turn) + Writhe

or

Linkage Number = #of Twists + Writhe

this is usually abbreviated as

Linkage = Twist + Writhe

L = T + W

For example, if we have a circularly closed DNA molecule with a length of 5300 base pairs, and a preferred conformation of 10.6 basepairs per turn, can it achieve this conformation without having to introduce any supercoiling (i.e. writhe)?

Apparent linkage number (Twist) = (5300 base pairs) / (10.6 base pairs/turn)

Apparent linkage number (Twist) = 500

In other words, in order to achieve the desired conformation of 10.6 bp/turn in the helix, exactly 500 turns are required over the length of 5300 base pairs.

Linkage number = 500 + Writhe

We can have integral values for the linkage number, and we can certainly introduce 500, which would require no Writhe at all:

500 = 500 + 0

What is the DNA molecule was 5200 base pairs?

Apparent linkage number (Twist) = (5200 base pairs) / (10.6 base pairs/turn)

Apparent linkage number (Twist) = 490.6

We can introduce either 490 or 491 as a linkage number, but not 490.6. What happens if there is a linkage number of 490 in the DNA molecule?

Linkage number = Twist + Writhe

490 = 490.6 + Writhe

Writhe = -0.6

In this case, the DNA adopts a negative 0.6 supercoil (about 108° of a right-handed supercoil) which will increase the apparent linkage number from 490 to 490.6 (and achieve 10.6 basepairs per turn in the duplex).

How many basepairs per turn would there be in the DNA if the DNA was not able to adopt any supercoil structure for this length of DNA with a linkage number of 490?

Linkage number = Twist + Writhe

490 = Twist + 0

Twist = 490 turns

Twist = (5200 base pairs) / (bp/turn) = 490 turns

bp/turn = 5200 base pairs/490 turns

bp/turn = 10.61

There are slightly more than 10.6 basepairs per turn in the DNA

A small circularly closed genome

The Simian Virus 40 (SV40) genome is a circular, closed, double stranded DNA genome. For the purposes of this discussion, it has 5300 bases. We expect that under physiological conditions the DNA will exhibit 10.6 base pairs per turn (i.e. one Twist = 10.6 bp/turn). In this case, with no Writhe, the Linking number would be:

Linking number = 5300 bp/(10.6 bp/turn) + 0

Linking number = 500 turns

i.e. we would expect 500 360° turns of the DNA strands over the length of the circular genome.

- This form (with 10.6 base pairs per turn) with no Writhe represents the "standard", or undistorted, DNA helix.
- This is also known as the **"relaxed"** form of DNA, and the duplex could physically be laid out **flat** on a surface because it needs no Writhe to achieve the preferred value of 10.6 basepairs per twist:

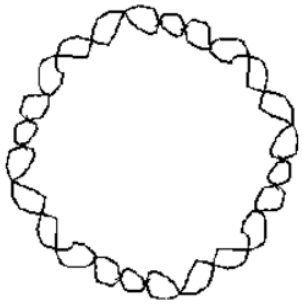


Figure 1.6.2: *Standard DNA helix*

However, when the replication of SV40 is initially completed it is observed that there remains an open duplex region in the DNA:

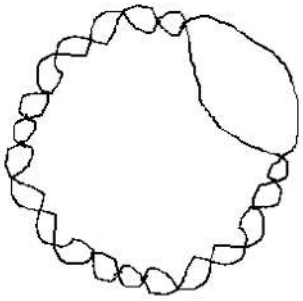


Figure 1.6.3: *DNA with open region*

The result is that there are about 475 turns of the helix within the duplex DNA (i.e. the Linking number = 475).

- The DNA is said to be **underwound**.
- An open area is energetically unfavorable.
- *The covalently closed molecule **cannot** adjust for this by increasing the Linking number*. That is, it cannot **spontaneously** break one or both strands of the duplex, introduce another 25 turns into the duplex (increase the Linking number by 25) and re-ligate the duplex.

The DNA has **three** choices:

1. It can adjust the number of **basepairs per turn** throughout the molecule from a desired 10.6 bp/turn to 11.2 bp/turn (i.e. 5300 bp/475 turns). (NOTE: an **increase** in the number of basepairs per turn will **decrease** the twist value; **underwound** DNA has a greater number of basepairs per turn).
2. The DNA can coil up into a "supercoil" topology and maintain the desired twist value (10.6) with the given linking number (475 in this case).
3. The duplex can exist with a twist of 10.6 bp/turn for most of the structure, and then have **a region with zero twist** (not necessarily a melted duplex). This is quite unfavorable due to the geometry required of bond angles.

Thus for the 5300 bp SV40 genome, with a Linking number of 475, to maintain a value of 10.6 bp/twist, a total of 25 negative supercoils (Writhe=25) are needed:

475 = (5300/10.6) + Writhe

-25 = Writhe

- That is, 25 **negative supercoils** (twenty five 180° turns of the DNA duplex, **right handed** as you look down the supercoiling).

Topoisomerases

The enzymes that control DNA topology are critical to DNA replication and transcription.

- As the replication fork opens up, the region of the duplex in front of the fork becomes ***overwound*** - i.e. it has *fewer basepairs per turn.*

- The linking number has not changed, but the length of DNA which contains all the turns is effectively shorter.
- To maintain 10.6 bp/turn in that region, *the DNA will adopt positive supercoils*.

For example, during the early stages of SV40 replication, the duplex around the origin of replication may initially melt (open up) a region of 750 bases. Since the Linkage number (500) is unchanged, it is effectively distributed over only:

5300 - 750 = 4550 bases.

Assuming no supercoiling has been introduced:

500 = 4550 basepairs / (X basepairs/twist) + 0

= 9.1 basepairs/twist

Thus, if no supercoiling is introduced, *the DNA must adopt a conformation of 9.01 base pairs/twist of the helix within the region ahead of the replication fork*.

- This is energetically unfavorable, and one option for the DNA is to adopt a ***supercoiled*** configuration to achieve 10.6 bp/twist:

500 = (4550/10.6) + Writhe

70.8 = Writhe

- *Thus, movement of the growing fork causes the DNA to adopt positive supercoils*.
- In this case the DNA has adopted 70.8 left handed supercoils (180° each).
- *Twist (=basepairs * [twist/basepair]) and Writhe are both real numbers*.

Type I Topoisomerase

- Type I topoisomerases cut one strand of the DNA (i.e. it "nicks" the DNA duplex).
- The 5' phosphate of the nicked strand is covalently attached to a tyrosine in the protein.
- The 3' end of the nick then passes once through the duplex.
- ***The nick is then resealed, and the linkage number is change by a value of +1.***
- This can therefore result in the removal of **a single negative supercoil**.

In *E. coli*, type I topoisomerase *can only relieve negatively supercoiled DNA* (negative supercoiling is the end result of newly replicated DNA genome). In eukaryotes, type I topoisomerase can also relieve positively supercoiled DNA.

The *net result* of *E. coli* topoI can be diagrammed as follows:

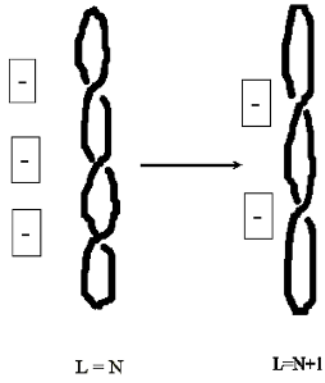


Figure 1.6.4: *E. Coli Topoisomerase I*

Type II Topoisomerases

- Type II topoisomerases actually cleave the duplex DNA in changing the linkage number.
- Type II topoisomerases can *convert a single positive supercoil into a negative supercoil*.
- ***Thus the linkage number is reduced by two (-2) in a single step.***
- Type II topoisomerases are involved in both decatenation of daughter chromosomes, and ***relieving the positive supercoiling ahead of the replication fork***.

- *E. coli* DNA **gyrase** is an example of a type II topoisomerase.

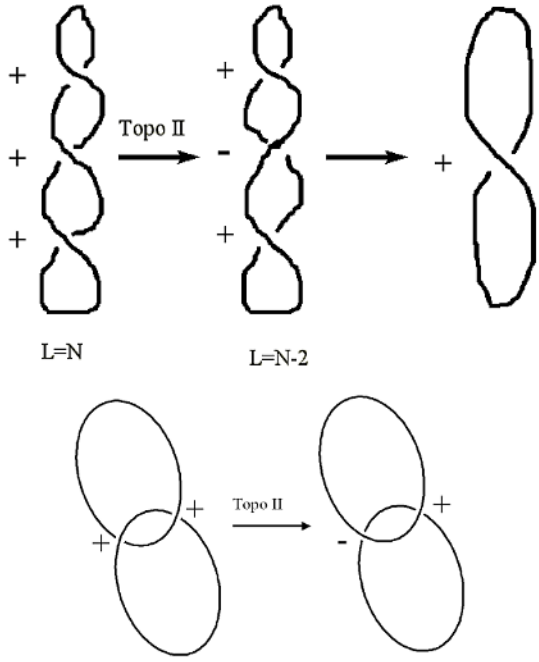


Figure 1.6.5: *Type II Isomerase activity*

This page titled 10.1.6: DNA Supercoiling and Topoisomerases is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **1.6: DNA Supercoiling and Topoisomerases** by Michael Blaber has no license indicated.

SECTION OVERVIEW

10.2: Bacteria

10.2.1: The Bacteria - An Introduction

10.2.2: Bacterial Growth and Reproduction

10.2.3: Extrachromosomal elements, plasmids, selectable markers

10.2.4: Central Dogma and the Genetic Code

10.2.5: Gene and Operon

10.2.6: The lac Operon; CAP site; DNA footprinting

10.2.7: Transcriptional Regulation- Transcription termination, the trp operon

10.2.8: mRNA Translation

This page titled 10.2: Bacteria is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

10.2.1: The Bacteria - An Introduction

Bacteria evolved some 600 million years ago, and were probably responsible for the production of the earth's atmosphere (cyanobacteria). Bacteria were discovered in the 17th century after the development of the microscope.

- Single cell organism
- Widely dispersed in the environment
- Invisible to naked eye, but discernible by their actions - milk sours, wounds become septic, meat putrefies, etc.
- *Prokaryotic* type cells (other organisms are *eukaryotic* type cells)

Major differences between prokaryotic and eukaryotic cells

Prokaryotic cells	Eukaryotic cells
No nuclear membrane: chromosome(s) in direct contact with cytoplasm	Chromosomes are enclosed in a double layered nuclear membrane
Simple chromosome structure	Complex chromosome structure; DNA associated with histone proteins
Cell division does not involve meiosis	Cell division involves mitosis and meiosis
If present, cell walls contain *peptidoglycan*, no cellulose or chitin	If present, cell walls contain *cellulose* or *chitin*, never peptidoglycan
No mitochondria or chloroplasts	Mitochondria usually present, chloroplasts in photosynthetic cells
Cells contain ribosomes of only one size	Cells contains two types of ribosomes, one in cytoplasm, and smaller type in mitochondria
Flagella, if present, have a *simple* structure	Flagella, if present, have *complex* structure

Note: bacteria are *microorganisms*, but not all microorganisms are bacteria. Algae, fungi, lichens, protozoa, viruses and subviral agents are all microorganisms (with most of these being eukaryotic type cells

Bacterial activities

Pathogenic (disease causing) bacteria:

- *Cholera* (vomiting, profuse diarrhea)
- *Botulism* (muscle paralysis)
- *Tetanus* (uncontrollable contractions of skeletal muscle)
- *Staphylococcal* food poisoning (vomiting, diarrhea)
- *Shiga toxin* (verotoxin) (classic dysentery)
- *Typhoid* (septicemia: bacteria in blood, destruction of host tissue)
- *Oroya fever* (Bartonella bacilliformis - destroys red blood cells)
- *Endotoxic shock* (lipopolysaccharide cell wall component causes release of host inflammatory agents leading to shock and death)
- *Reactive arthritis* (response in some people to Salmonella infection)

Most bacteria do no harm to humans, and can be quite useful:

- Antibiotic production
- Enzyme additives for detergents
- Insecticides
- Production of biodegradable plastics
- "Biomining" - leaching of metals from low grade ores
- Uses in food industry
  - Butter
  - Cheese
  - Yogurt
  - Vinegar
  - Cocoa
  - Coffee
- Soil fertility

Classifying and Naming of bacteria

Differences between bacteria can include

- shape
- size

- structure
- chemical activities
- required nutrients
- form of energy required
- required environment
- reaction to certain dyes

Family, genus, species, strain

Bacteria in the same *Family*, in general would have:

- similar structure
- use the same form of energy
- react similarly to certain dyes

Bacteria in the same *Family* may be divided into different *Genera* based on differences in

- chemical activities
- nutrient requirements
- conditions for growth
- shape and size (to some extent)

Strains of bacteria are bacteria of the same species, but with some subtle difference (maybe a single mutational difference)

Latin binomial name

- Name of the genus, capitalized.
- Followed by name of species, lower case
- Italicized. Sometimes the genus name is abbreviated to a single letter (with a period)

Escherichia coli
E. coli

- Strain name follows, usually in parentheses. In the vernacular, the strain name is commonly used to identify the bacteria

E. coli (JM101)
E. coli (CJ236)
"Can I borrow some JM101?"

Some Characteristics of Bacteria

Shape

- Rounded or spherical cells - *cocci* (singular: *coccus*)
- Elongated or rod-shaped cells - *bacilli* (singular: *bacillus*)
- Rigid spirals - *spirilla* (singular: *spirillum*)
- Flexible spirals - *spirochetes* (singular: *spirochete*)

Note

There is a genus of bacteria called Bacillus. Some bacillus shaped bacteria belong to the genus Bacillus, some do not.

Size

- Bacteria are usually measured in micrometers ($1x10^{-6}$ m)
- The smallest bacteria are about 0.2 micrometers (*Chlamydia*)
- The largest bacteria are about 600 micrometers (*Epulopiscium fishelsoni*. - inhabits the gut of a fish)
- "Average" bacteria are 1-10 micrometers (note: limit of resolution of the light microscope is about 0.2 micrometers)

A "generalized" bacterium:

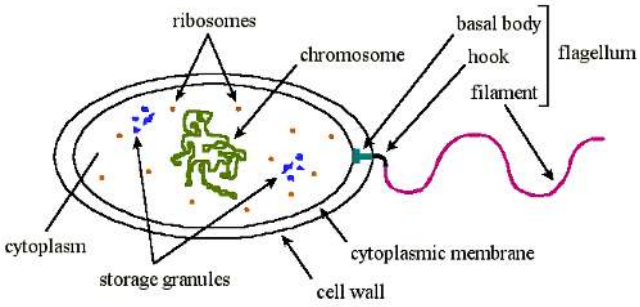


Figure 2.1.1: *General bacterium diagram*

- The cell's DNA is extensively folded to form a body called the *nucleoid*
- The *cytoplasm* fills the interior of the cells, and bathes the nucleoid
- *Storage granules* contain a reserve of nutrients - typically polymeric forms of b-hydroxybutyrate and phosphate. Poly-b-hydroxybutyrate is the basis of a biodegradable plastic (Biopol)

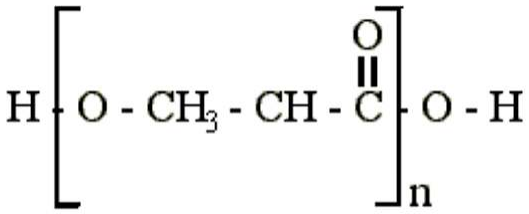
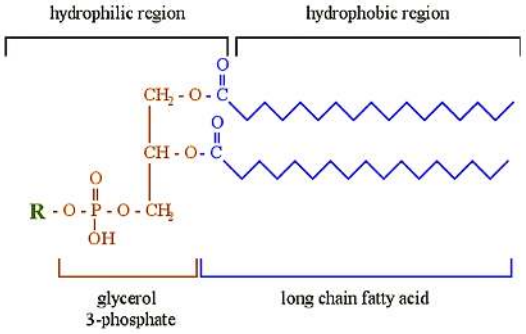


Figure 2.1.2: *Poly-b-hydroxybutyrate*

- The nucleoid, ribosomes, cytoplasm and storage granules are bounded by a membranous sac, the *cytoplasmic membrane* (*cell membrane*, or *plasma membrane*)
- The outermost layer is a tough *cell wall*. Together, the plasma membrane and cell wall are called the *cell envelope*
- The region between the plasma membrane and the cell wall is called the *periplasmic space*
- The *flagellum* is used for motility

Cytoplasmic membrane

- ***lipid bilayer***, 7-8 nm thick, with protein molecules partly or completely embedded
- The inner and outer layers are *hydrophilic*, while the interior of the bilayer is *hydrophobic*
- In *E. coli* the main lipid is phosphatidylethanolamine; minor lipid components include phosphatidylglycerol and diphosphatidylglycerol



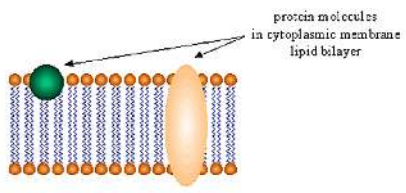


Figure 2.1.3: *Phospholipid bilayer*

The cytoplasmic membrane proteins include:

- enzymes involved in the synthesis of the cell wall peptidoglycan
- transport proteins (translocated ions and molecules across the cytoplasmic membrane
- proteins of energy converting systems (ATPases and electron transport chains)
- "sensory" proteins, which detect changes in cell's external environment

The cytoplasmic membrane is not freely permeable to most molecules

- some small uncharged molecules (O_2, CO_2, NH_3, H_2O) can freely pass through
- charged ions typically cannot pass across the membrane, and must be *transported* (with the expenditure of energy)

If the cell wall is removed, what remains of the cell is called the *protoplast*

- can survive (in a test tube) and carry out most normal cell processes
- quite sensitive to osmotic shock - if placed in pure water it will swell (as water enters the cell to balance the osmotic force) and rupture (*osmotic lysis*)
- In an intact cell the cell wall prevents the protoplast from swelling and undergoing osmotic lysis
- The cell wall also determines the shape of the bacteria - all protplasts are spherical, regardless of the shape of the intact bacteria

The Cell Wall

Among the *Eubacteria* (*Kingdom* of all bacteria excluding the *archebacteria*, which are typically halophiles and thermophiles) ***there are only two major types of cell wall***

- They can be identified by their reaction to certain dyes (characterized by Christian Gram in 1880's):

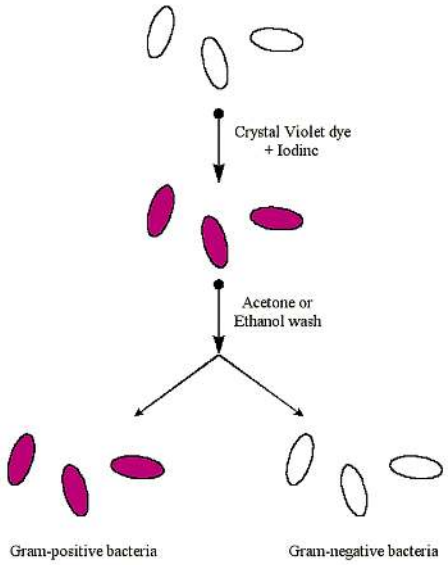


Figure 2.1.4: *Gram positive and Gram negative bacteria*

Gram positive type cell wall

- relatively thick (30-100 nm)
- 40-80% of the wall is made of a tough complex polymer called peptidoglycan (linear heteropolysaccharide chains cross-linked by short peptides)

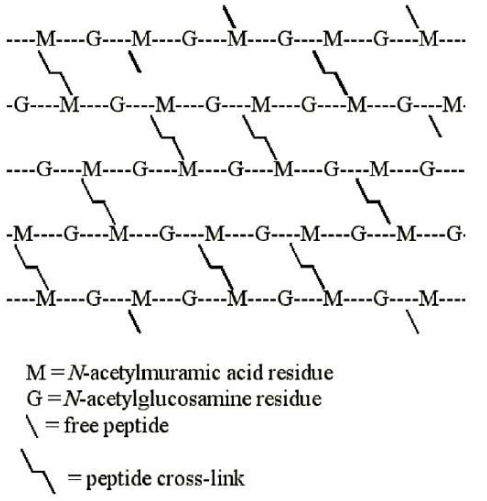


Figure 2.1.5: *Gram positive cell wall*

- The cell wall of a gram-positive cell is a multi-layed network which appears to be continually growing by the addition of new peptidoglycan at the inner face, with concommitant loss at the outer surface

Gram-negative type cell wall (e.g. E. coli)

- thinner than gram-positive type cell wall (only 20-30 nm thick)
- has distinctly layered appearance
- inner region consists of a monolayer of peptidoglycan
- outer layer of cell wall is *essentially a protein containing lipid bilayer*
  - inward facing lipids are phospholipids
  - outward facing lipids are macromolecules called lipopolysaccharides

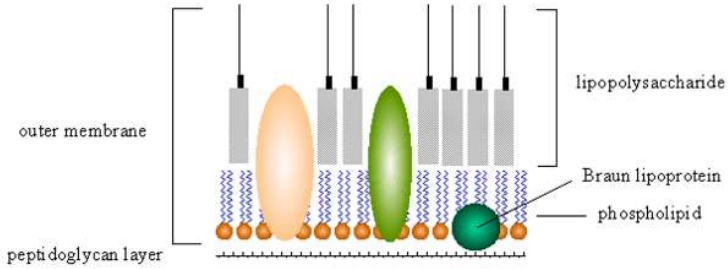


Figure 2.1.6: *Gram negative cell wall*

- half the mass of the outer membrane consists of proteins
  - the ***Braun protein***, which is covalently linked to the peptidoglycan layer
  - transport proteins
  - ***porins*** - molecules which span the outer membrane to create a 'pore' through the membrane. These pores allow certain molecules and ions to pass through the outer membrane (e.g. *ompC*, *ompF* proteins)
- adjacent outer lipopolysaccharides are held together by electrostatic interactions with divalent metal ions (Ca^{2+}, Mg^{2+})
  - the addition of chelating agents (e.g. EDTA) can disrupt these interactions and weaken the outer membrane
- lysozyme (produced by phage lambda, for example) can cleave the saccharide links in the inner peptidoglycan layer

How to lyse a gram-negative bacteria (e.g. *E. coli*):

1. Add a chelating agent of divalent metals (e.g. EDTA) to disrupt outer membrane lipopolysaccharides
2. Add lysozyme to break up peptidoglycan layer
3. *cell wall is now structurally weakened and cannot protect the protoplast from osmotic shock*
4. osmotically shock the cell to disrupt protoplast and release cytoplasmic contents (i.e. high osmotic shock using sucrose solution; low osmotic shock using pure water),
5. or use mechanical shear/cavitation (French Press, Menton Gaulin press)

Human microflora: some of the bacteria commonly associated with the human body

Location	Genus
Colon	*Bacteroides, Clostridium, Escherichia, Proteus*
Ear	*Corynebacterium, Mycobacterium, Staphylococcus*
Mouth	*Actinomyces, Bacteriodes, Streptococcus*
Nasal passages	*Corynebacterium, Staphylococcus*
Nasopharynx	*Streptococcus, Haemophilus (e.g. H. influenzae)*
Skin	*Propionibacterium, Staphylococcus, Others (personal hygiene, environment)*
Urethra	*Acinetobacter, Escherichia, Staphylococcus*
Vagina (adult, pre-menopausal)	*Acinetobacter, Corynebacterium, Lactobacillus, Staphylococcus*

This page titled 10.2.1: The Bacteria - An Introduction is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **2.1: The Bacteria - An Introduction** by Michael Blaber has no license indicated.

10.2.2: Bacterial Growth and Reproduction

Flagella and motility

- *monotrichous* flagella - the bacterial cell has a single flagella
- *peritrichous* flagella - the bacterial cell has several flagella which are located at various sites on the cell surface (e.g. *E. coli*)

Motility is due to the presence of one or more flagella.

- in peritrichous flagellate bacteria the flagella rotate independently of one another
  - 95% of the time the flagella rotate counterclockwise
  - 5% of the time the flagella switch directions and rotate clockwise
  - When the flagella are all rotating counterclockwise, the flagella are bundled together and the bacteria travels in a straight line (i.e. it swims)
  - When one flagella switches direction the bundle disassociates, and the bacteria tumbles
  - alternative swimming and tumbling results in a three-dimensional random walk
- chemoattractants and repellents can interaction with receptor proteins in the cell envelope, which in turn influence the rate of tumbling when the cell is moving in a given direction

Growth and Reproduction

Essential requirements for growth include:

- supply of suitable nutrients
- source of energy
- water
- appropriate temperature
- appropriate pH
- appropriate levels (or absence) of oxygen

Nutrients

Cells need a source of:

- carbon
- nitrogen
- phosphorous
- sulfur
- other trace materials

Although a given bacteria typically uses a limited range of compounds, bacteria as a group can utilize a wide range of compounds as nutrients:

- sugars and carbohydrates
- amino acids
- sterols
- alcohols
- hydrocarbons
- methane
- inorganic salts
- carbon dioxide

Energy

Energy is needed for

- essential chemical reactions

- uptake of nutrients
- flagellar motility

Phototrophic vs chemotrophic bacteria

- *phototrophic* - energy derived from light source
- *chemotrophic* - energy is obtained by processing chemicals from the environment

Water

- 80% of the mass of typical bacteria is water
- Water is needed for growth and reproduction
- Dessication (extreme lack of water) is tolerated to different degrees by different bacteria

Temperature

- Growth proceeds most rapidly at the *optimum growth temperature* for a particular bacteria (and decreases as temperature is raised or lowered from this optimum)
- For any bacteria, there is a *minimum* and *maximum* temperature beyond which growth is not supported

Thermophilic bacteria

- *optimum* growth temperature is >45°C
- occur in compost piles, hot springs and ocean floor hydrothermal vents
- *Pyrodictium* have an optimum growth temperature of 105°C

Mesophilic bacteria

- *optimum* growth temperatures between 15 and 45°C
- live in a wide range of habitats
- since the human body is 37-42°C, human pathogenic bacteria are *mesophiles*

Psychrophilic bacteria

- optimum at 15°C or below
- minimum temperature of 0°C, or less
- maximum temperature of 20°C
- occur in polar seas

pH

- most bacteria grow optimally near neutral pH (7.0)
- *acidophiles* have an optimum pH with is more acidic (*Thermoplasma acidophilum*, found in hot springs, prefers pH 0.8-3, and will not grow at neutral pH)
- *alkalophiles* have an optimum at higher (alkaline) pH ranges (*Exiguobacterium aurantiacum*, found in natural alkaline lakes, prefers pH 8.5-9.5)

Oxygen

- bacteria which must have oxygen for growth are termed *obligate aerobes*
- bacteria which can grow only in the absence of oxygen are termed *obligate anaerobes* (e.g. environment which are isolated from the atmosphere; e.g. river mud, and within the intestines, for example)
- bacteria which normally grow in the presence of oxygen, but which can manage to grow in its absence, are termed *faculative anaerobes*
- conversely, bacteria which normally grow anaerobically, but which can manage to grow in the presence of oxygen, are termed faculative *aerobes*

Inorganic ions

All bacteria need low concentrations of certain inorganic ions in order to functions, e.g.

- iron for cytochromes (energy metabolism), and certain enzymes

- magnesium for cell wall stability
- manganese and nickel in metabolic enzymes

- high concentrations usually inhibit growth (e.g. salt has been used in the preservation of pork, beef and cod)
- some bacteria, *halophiles*, grow only in the presence of high concentrations of certain salts (e.g. sodium chloride). *Halobacteriaceae* grow only in the presence of 3-4 M NaCl. This amount of salt is needed to maintain the structure of the cell wall and internal molecular assemblies (e.g. ribosomes).

Growth in a single cell (e.g. *Escherichia coli* -a gram negative bacillus)

The cycle of events in which a cell grows, and divides into two daughter cells, is called *the cell cycle*.

"Slow Growth"

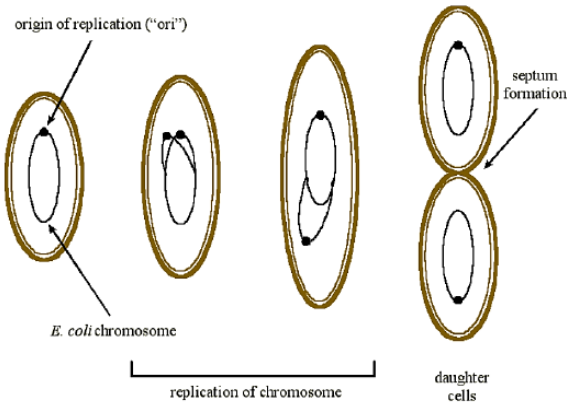


Figure 2.2.1: *Replication for slow growth*

- Replication begins at a specific place on the chromosome - the *origin* or "ori" region.
- During "slow growth" each new daughter cell contains exactly one chromosome, because a new round of chromosomal replication does not begin until after completion of cell division

The cell division cycle can be thought of as a linear sequence of three periods: ***I***, ***C*** and ***D***

- ***C*** is the period during which chromosomal replication occurs
- ***D*** is the period in which the septum forms, and *cell division occurs at the end of the* ***D*** *period*
- ***I*** is the period between each successive initiation of chromosomal replication

For the above type of slow growth, the relationship between these three periods is as follows:

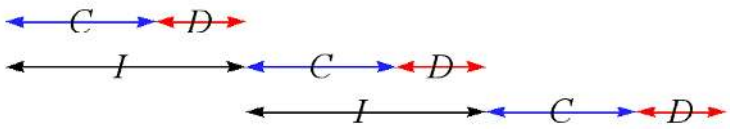


Figure 2.2.2: *Periods of cell cycle for slow growth*

- ***I*** is also known as the ***doubling time*** of the bacterial growth

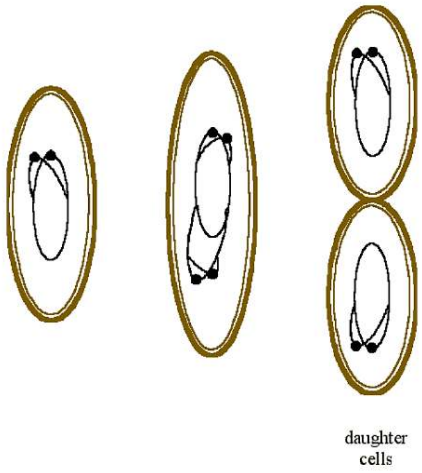


Figure 2.2.3: *Replication for rapid growth*

- A new round of chromosome replication begins *before* cell division occurs

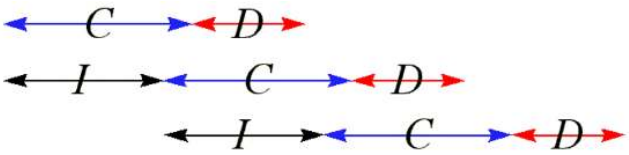


Figure 2.2.4: *Cell cycle for rapid growth*

- Each daughter cell has the equivalent of about 1 ½ chromosomes
- In rapidly growing *E. coli* cells the *C* period is about 42 minutes and *D* is about 25 minutes.
- The maximum doubling time for *E. coli* is about 20 minutes

- "***medium***":any solid or liquid specially prepared for bacterial growth
- "***culture***": a liquid or solid medium containing bacteria which have grown (or are growing) in or on that medium
- "***incubation***": the process of maintaining a particular temperature (and/or other desirable conditions) for bacterial growth
- "***innoculation***": the initial process of adding the cells to the medium

Growth on a solid medium

- Liquid solution of nutrients plus 1% *agar* (the preferred form of *Jello* in Asia. A polysaccharide extracted from seaweed; as opposed to Western *Jello* which is a protein extracted from the hooves of large farm animals) forms solid media

Typical Solid Media Recipe (1 liter). The media here is commonly called "Luria Broth", or "LB". It is named after one of the scientists who developed it (not "Dr. Broth").

- Yeast extract 5 g (nucleic acids, cofactors, inorganic salts, carbohydrates)
- Tryptic digest of casein (milk protein) 10g (peptides and amino acids)
- NaCl 10g (note: final concentration is thus 0.17 M, or close to *physiological*)
- Agar 10g
- Water (bring volume up to 1.0 liter)
- Autoclave, pour into petri dish, let cool

An individual bacterial cell will divide and eventually become a visible mass of cells known as a *colony*

If instead of a single cell, the solid media is initially populated with a large number of cells, *confluent growth* or a *lawn* of bacteria will be visible

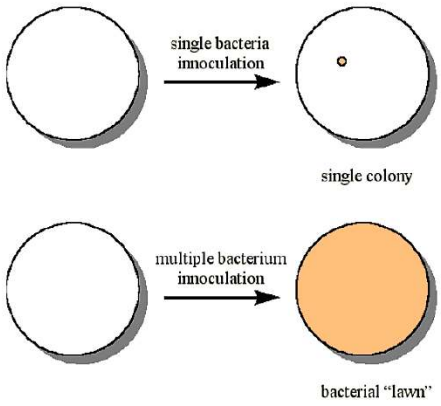


Figure 2.2.5: *Growth on solid medium*

"Streaking" solid media plates

- bacteria can be introduced onto solid media by a sterile transfer tool, such as a wire loop (nichrome wire), or autoclaved toothpick, which has been dipped into a bacterial culture
- such a transfer contains *thousands* of individual bacterial cells
- it is desirable to grow colonies from individual cells rather than from a large population

This is done to avoid the takeover of the strain by potential wild-type revertants

- "streaking" is a method to isolate individual cells for growth on solid media from an inoculation which originally contains thousands of cells

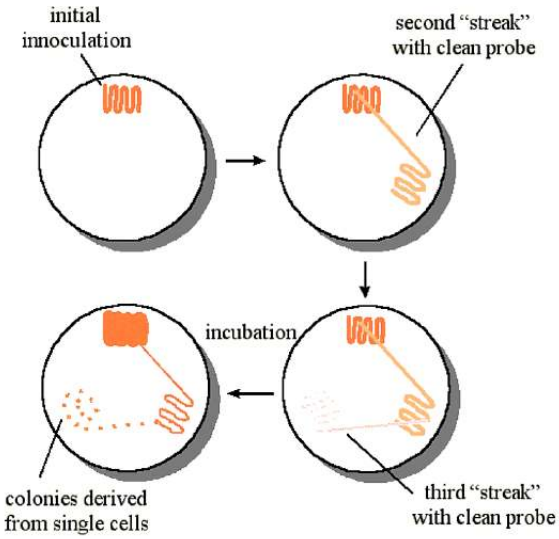


Figure 2.2.6: *Streaking*

Growth in liquid medium

- No agar
- Use Erlenmeyer flask, or fermenter
- If necessary, aerate, agitate
- progeny will be dispersed throughout medium (diffusion or locomotion)
- as the cell density increases, the media becomes *turbid*
- number of cells plotted versus time will yield a *growth curve*

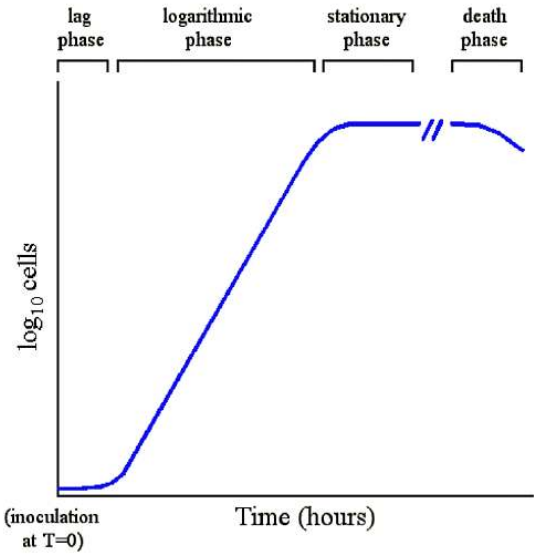


Figure 2.2.7: *Growth curve in liquid medium*

- "***lag phase***" after inoculation cells are becoming acclimated to the new environment (temp, nutrients, etc.)
- "***log phase***" cells have adapted and are dividing at a constant rate (i.e. the maximum for the species under the given conditions of temp, pH, nutrients, oxygen, etc.)
- "***stationary phase***" cell growth ceases as nutrients are exhausted and/or waste products build up in the media
- "***death phase***" number of viable (living cells) in the stationary phase culture decreases (usually due to toxicity of waste products)

Cell density can be conveniently monitored using the absorbance of visible light (usually at 600 nm)

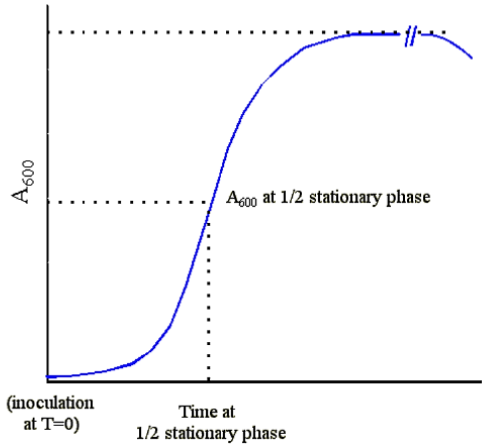


Figure 2.2.8: *Cell density curve*

Different media will result in different growth rates and different stationary phase densities

- Rich media will have short (<1.0 hour) doubling times and will result in higher cell densities at stationary phase
- Minimal media will exhibit slow growth (doubling times ~1.0 hour at 37°C) and low final densities

- Efficient agitation and aeration can increase final cell densities (fermenters may achieve higher densities than shaker flasks).

	Minimal	LB	Terrific
Phosphate salts	16g	-	12g
Ammonium salts	1g	-	-
Magnesium salts	0.1g	-	-
Glucose/glycerol	4g	-	4g
Sodium Chloride	0.5g	10g	-
Enzymatic digest of casein (milk protein)	-	10g	12g
Yeast extract	-	5g	24g
Approx doubling time (min)	60	45	30
Stationary phase density (A_{600})	3	7	15

Let's take a look at some raw data from a culture of E. coli growing in a fermenter. The absorbance at 600nm was recorded at various time intervals:

Time (minutes)	A_{600}
0	0.09
18	0.102
78	0.124
142	0.253
205	0.487
255	1.02
322	1.98
378	3.95
446	5.88
504	6.76
564	7.2

`

The growth curve looks like this:

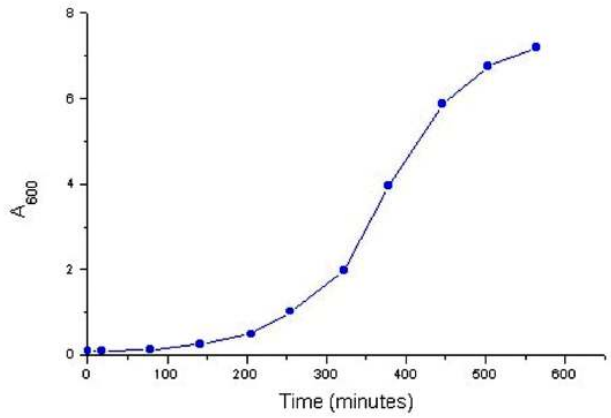


Figure 2.2.9: *Sample growth curve*

In order to calculate the doubling time we need to know the region of the growth curve for which the growth is *logarithmic*. We can evaluate this by plotting the absorbance data on a logarithmic ($\log_{10}$) scale. In this case, our data will look like this:

Time (minutes)	**A_{600}**	**$\log_{10} A_{600}$**
0	0.09	-1.05
18	0.102	-0.99
78	0.124	-0.91
142	0.253	-0.60
205	0.487	-0.31
255	1.02	0.01
322	1.98	0.30
378	3.95	0.60
446	5.88	0.77
504	6.76	0.83
564	7.2	0.86

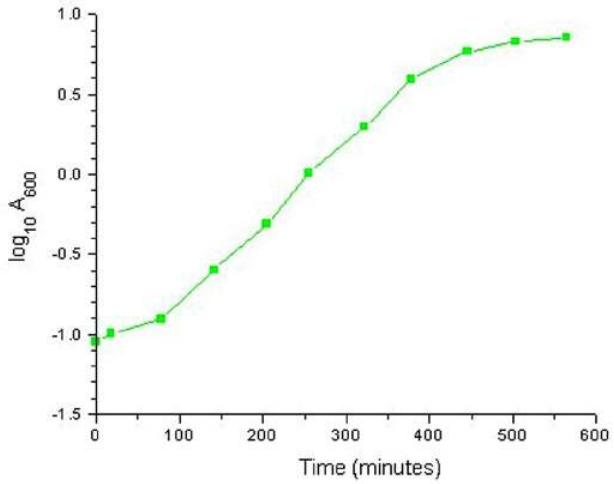


Figure 2.2.10: *Sample logarithmic growth curve*

In this case, the data appears to have an initial lag period followed by logarithmic growth until about 400 minutes, then the rate slows. In other words, the $\log_{10}$ curve appears linear over the time period 100 - 400 minutes, or so.

We can conveniently determine the doubling time by replotting the data over this period and converting the A_{600} into $\log_2$ values.

Note

$\log_N(X) = \log_{10}(X)/\log_{10}(N)$

Time (minutes)	A_{600}	$\log_{10} A_{600}$	$\log_2 A_{600}$
0	0.09		-1.05
18	0.102	-0.99	
78	0.124	-0.91	-3.01
142	0.253	-0.60	-1.98
205	0.487	-0.31	-1.04
255	1.02	0.01	0.029
322	1.98	0.30	0.99
378	3.95	0.60	1.98
446	5.88	0.77	
504	6.76	0.83	
564	7.2	0.86	

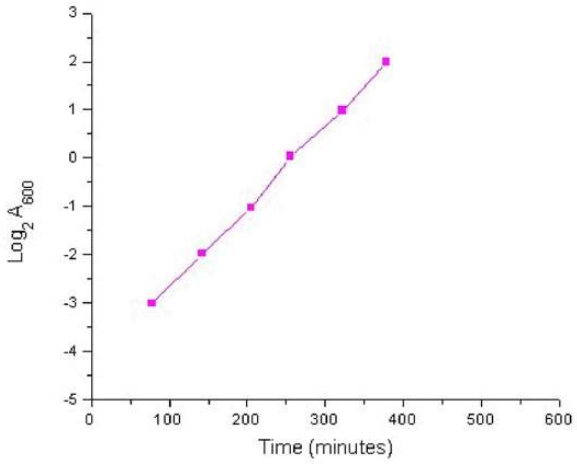


Figure 2.2.11: *Sample log_2 growth curve*

If we plot a straight line through these points the slope will give us the rate of change of the $\log_2 A_{600}$ values as a function of time:

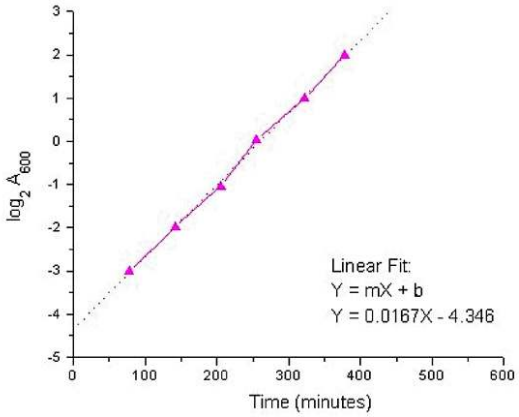


Figure 2.2.12: *Linear fit for sample log_2 growth curve*

- Thus, for this culture, the growth (slope) can be described as a rate of 0.0167 $\log_2 A_{600}$/min.
- The inverse of the slope will therefore tell us how many minutes it takes for the culture to increase its density by 1 $\log_2 A_{600}$ absorbance units.
- Since it is $\log_2$, a change of 1 absorbance units means the absorbance has doubled.

Therefore, the inverse of the slope for the log_2 plot gives us the time it takes for the culture absorbance to double (i.e. the doubling time)

For this experiment, the doubling time is 1/0.0167 or **59.9 minutes**. This suggests that the media is probably not very rich (maybe like minimal media), however, the final absorbance is higher than that expected for minimal media. So, possibly the media LB, but the growth was done at a lower temperature (i.e. leading to a slower growth rate).

Important parameters to determine:

- A_{600} at stationary phase
- A_{600} at ½ stationary phase (***usually the time point chosen to stimulate bacteria to produce recombinant proteins***)
- *Doubling time* (plot $\log_2$ of A_{600} over logarithmic range of growth, take inverse slope)

This page titled 10.2.2: Bacterial Growth and Reproduction is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **2.2: Bacterial Growth and Reproduction** by Michael Blaber has no license indicated.

10.2.3: Extrachromosomal elements, plasmids, selectable markers

- Including an origin of replication (i.e. the *E. coli* ***oriC*** region) into a circular DNA molecule is a mechanism to have an **extrachromosomal element** in the prokaryotic cell.

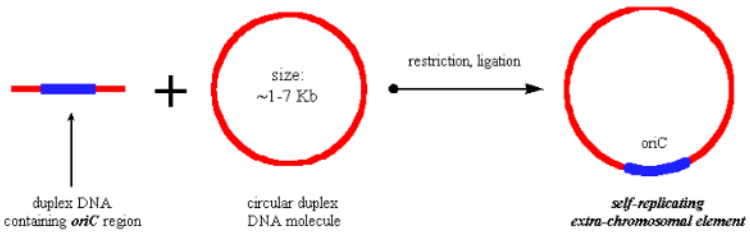


Figure 2.3.1: *oriC Extrachromosomal element*

- Such an extrachromosomal element is called a ***plasmid***, or ***vector***
- The plasmid uses the host cell machinery (i.e. polymerases, helicases, dNTP's etc.) to direct replication.

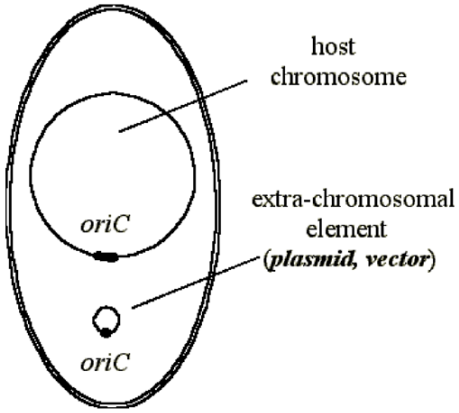


Figure 2.3.2: *Vector/Plasmid*

- However, since the added work of replicating the extrachromosomal element is a load on a cell, ***it will be out-competed by other cells which do not contain the plasmid***.
  - Since in prokaryotic cells the segregation of plasmids is a random event, daughter cells can arise which do not contain the plasmid and these grow faster (out-compete) the parent cell.
  - In other words, in the absence of other pressures, after a period of time the population of cells in a culture will be those which have "lost" the plasmid.

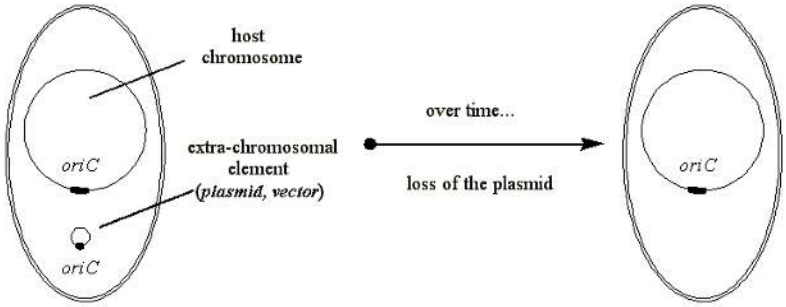


Figure 2.3.3: *Loss of plasmid*

- In organisms with more than one chromosome (eukaryotes) there are a variety of mechanisms to ensure that proper segregation of chromosomes occurs, i.e. to make sure that daughter cells contain equal numbers of all the chromosomes.
- One basic mechanism is that each chromosome contains **essential genes**, and if these are lost, the cell cannot survive.

Drug resistance

- By far the most common approach to the maintenance of plasmids is through the incorporation of drug resistance genes.
  - These are also known as **selectable markers,** i.e. we can select for their presence by including ***antibiotics*** in the growth media.

Ampicillin

- Ampicillin binds to and inhibits a number of enzymes in the bacterial membrane that are involved in the synthesis of the ***gram negative*** cell wall.
  - Therefore, proper cell replication cannot occur in the presence of ampicillin.
- The ampicillin resistance gene (***amp^r***) codes for an enzyme (***b-lactamase***) that is secreted into the periplasmic space of the bacterium where it catalyzes hydrolysis of the b-lactam ring of the ampicillin.
  - Thus, the gene product of the amp^r gene **destroys the antibiotic**.
- Over time the ampicillin in a culture medium or petri plate may be substantially destroyed by b-lactamase.
  - When this occurs, cell populations can arise which have "lost" the plasmid.

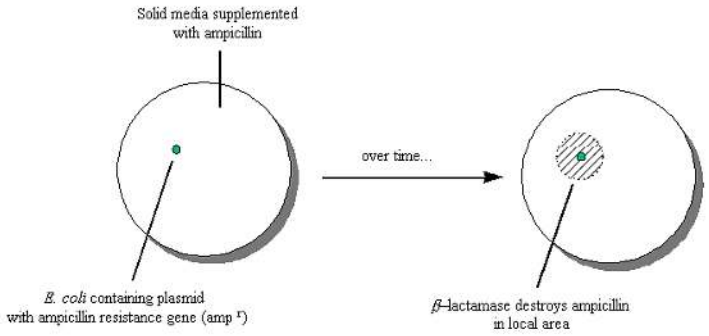


Figure 2.3.4: *Ampicillin resistance*

Tetracycline

- Tetracycline binds to a protein of the ***30S*** subunit of the ribosome and inhibits ribosomal translocation along the messenger RNA which codes for the protein (i.e. the drug interferes with normal translation or production of proteins).
  - The tetracycline resistance gene (tet^r) encodes a 399 amino acid outer membrane associated protein of *gram negative* cells that ***prevents the antibiotic from entering the cell***.
- Thus, this drug resistance gene ***does not destroy the antibiotic***. Pressure will be maintained throughout the cell culture process to keep the plasmid containing the drug resistant gene.

Chloramphenicol

- Chloramphenicol binds to the ribosomal ***50S*** subunit and inhibits protein synthesis.
- The chloramphenicol resistance gene (***Cm^r***) codes for a protein known at the *cat* protein.
  - The *cat* protein is a tetrameric cytosolic protein that, in the presence of acetyl coenzyme A, catalyzes the formation of hydroxyl acetoxy derivatives of chloramphenicol that are unable to bind to the ribosome.
  - Like with *ampicillin*, ***the Cm^r gene product destroys the antibiotic***.
  - Additionally, the expression of *cat* protein is influenced (down regulated) by the presence of glucose in the media.

Kanamycin and neomycin

- Bind to ribosomal components and inhibits protein synthesis.
- The Kan^r gene codes for a protein which is secreted into the periplasmic space and interferes with the transport of these antibiotics into the cell.
  - Like tetracycline resistance, the Kan^r gene does not destroy the antibiotic.

Colicin E1

- This is a member of a general class of substances known as ***bacteriocins***.
- *Colicin E1* causes lethal membrane changes in bacteria.
  - The drug resistance gene (*cea*) codes for a protein that interferes with the action of colicin in an unknown manner.

Prokaryotic plasmids

- In addition to making a plasmid by using the *E. coli OriC* region, there is a naturally occuring *E. coli* plasmid called the **ColE1 plasmid**
  - The ColE1 origin of replication is uni-directional (unlike *oriC*)
  - The replication from the ColE1 *ori* region does not require the associated proteins (e.g. *dnaA* protein) like *oriC*, (but it does require specific RNA molecules).
- Depending on the exact region of the ColE1 origin which is inserted into a circular dna molecule, the extrachromosomal element will be maintained with either a "low" or "high" copy number
- The ***rop*** gene near the ColE1 origin is involved in the regulation of replication.
  - If the ColE1 ori region includes this gene then the plasmid is maintained with an average copy number of 10-30 plasmids/cell. This is considered ***low copy*** number.
  - If the ColE1 ori region does not have the rop gene, then the resulting plasmid is maintained with an average copy number of 100-200 plasmids/cell. This is considered a ***high copy number*** type of plasmid.
- If the plasmid contains a gene coding for a protein (like drug resistance genes) copy number can influence the amount of such protein in the cell.

pBR322 (4.36 Kb)

- One of the original cloning plasmids.
- Constructed by ligating together:
  - the tetracycline resistance gene from plasmid pSC101
  - ColE1 and rop region from the ColE1 plasmid
  - the ampicillin resistance gene from the transposon Tn3
- Contains both ***ampicillin*** and ***tetracycline*** resistance genes (markers).
- Contains unique restriction sites inside and outside of these markers.
- Contains ***rop*** region near CoE1 ori , therefore, it has a low copy number (10-30)
- Numbering begins at the unique EcoR I restriction site (GAATTC). The first 'T' in this sequence is base number "1".

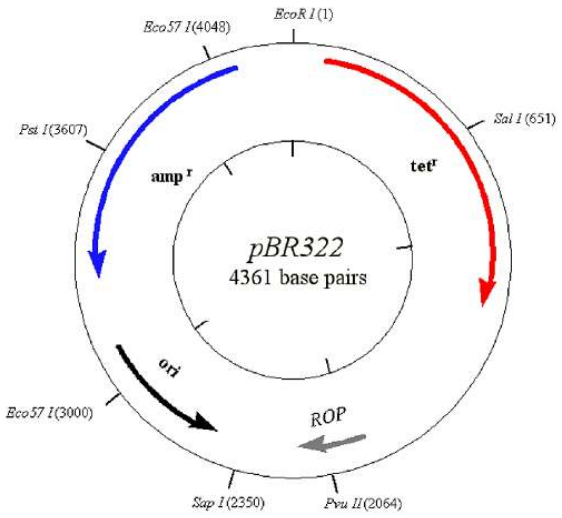


Figure 2.3.5: *pBR322*

The plasmid diagram:

- In the center is the plasmid name (usually starts with a lower case 'p') and the size in basepairs
- the inner ring provides ticks in 1 Kilobase (Kb) intervals to give an idea of the general location of parts of the plasmid
- the arrows indicate genes, markers, ori or replication, promoters, polylinkers, transcription terminators, and other important or functional items
- the outer ring usually indicates the location of unique, or limited number (usually <3), restriction endonuclease sites. Restriction enzymes which have more than three sites will not be indicated. Be aware that enzymes which do not cut at all will also not be listed!

PUC18/19 (2.69 Kb)

- Lacks the *rop* gene near the ColE1 ori region. Thus, this plasmid tends to accumulate in high copy number (100-200).
- This vector contains only the ampicillin resistance marker.
- This vector contains a ***polylinker*** region
  - A synthetic DNA sequence which contains a clustering of unique restriction enzyme recognition sites
  - Allows DNA fragments, generated by a variety of restriction endonuclease cleavages, to be inserted into the plasmid
  - pUC18 has the polylinker in one orientation
  - pUC19 has the same polylinker, but in the ***opposite*** orientation
  - Thus, ***fragments with unique restriction sites on each end can be inserted in a specific orientation***
  - The ***Pst I*** site in the *amp*^{r} gene was mutated to remove it. The ***EcoR I*** site at position (1) was mutated to remove it. ***This was done to make the restriction sites in the polylinker region unique***
- This vector also contains a *transcription promoter region* from the ***lac operon***, which allows foreign genes to be inserted and transcribed/translated.
  - The polylinker region is just downstream (3') to the lac promoter
  - Inserted genes can be transcribed from this promoter

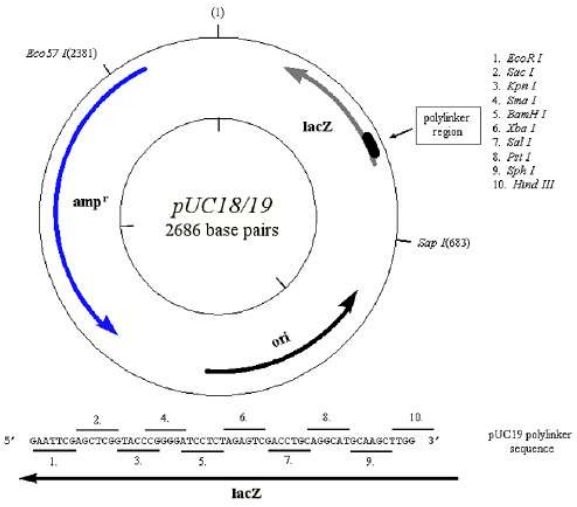


Figure 2.3.6: *pUC18/19*

This page titled 10.2.3: Extrachromosomal elements, plasmids, selectable markers is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **2.3: Extrachromosomal elements, plasmids, selectable markers** by Michael Blaber has no license indicated.

10.2.4: Central Dogma and the Genetic Code

The "Central Dogma"

We have seen how DNA, with the aid of specific polymerases and accessory proteins, is able to replicate. We have also seen how we can use this information to create autonomously replicating extra-chromosomal elements (i.e. **plasmids**). However, the real utility of such systems arises when we use them to create **proteins** of interest. To get to proteins we have to go through **RNA** first.

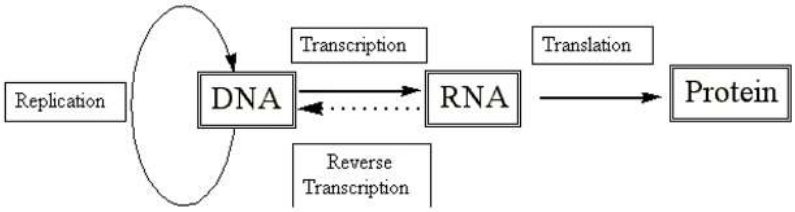


Figure 2.4.1: *Central dogma*

Structural features of RNA:

1. Similar to DNA except it contains a 2' hydoxyl group (**makes phosphodiester bond more labile than DNA**).
2. **Thymine** in DNA is replaced by **Uracil** in RNA

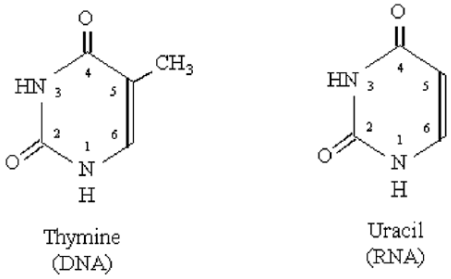


Figure 2.4.2: *Thymine vs. Uracil*

3. RNA's can adopt regular ***three-dimensional structures*** which allow them to function in the process of genetic expression (i.e. the production of proteins).

- This ability to adopt defined three dimensional structures which impart functionality places RNA in a unique class - somewhat akin to proteins, and different from DNA.
- For example certain RNA molecules, when folded, exhibit ***catalytic capacities*** (e.g. the cleavage of RNA molecules).
- The majority of RNA in cells is found in complexes with proteins. The most common example is ribosomes (involved in protein synthesis).

Transcription: the copying of DNA by an RNA polymerase to make RNA.

RNA polymerase:

- *Can initiate* a new nucleic acid strand given a template.
- DNA polymerases **cannot**; they require a **primer** (or more typically, an RNA polymerase to provide the primer).

Protein Synthesis

- Three kinds of RNA molecules perform different functions in the protein synthesizing apparatus:

1. *Messenger RNA* (mRNA) encodes the genetic information copied from DNA in the form of a sequence of bases that specifices a sequence of amino acids
2. *Transfer RNA* (tRNA) is part of the structural machinery which deciphers the mRNA code. They carry specific amino acids which are transfered to a nacent polypeptide according to the instructions contained within the mRNA.

3. *Ribosomal RNA* (rRNA) forms a complex with specific proteins to form the **ribosome** which is the key translational component

- the ribosome complexes with mRNA and directs appropriate tRNA's and the synthesis of the polypeptide bond.

Translation:

The process by which the information contained within a mRNA is used to direct the synthesis of the corresponding polypeptide.

The Genetic Code

How is the information for a polypeptide sequence stored within an mRNA molecule? There are twenty different common amino acids, but only four different bases in RNA (*A, C, G, and U*).

Base Arrangement	Possible Combinations
1	$4^1=4$
2	$4^2=16$
3	$4^3=64$
4	$4^4=256$

A triplet arrangement would seem to be the minimum possible combination necessary to code for the 20 different amino acids. Although, there are obviously going to be a lot of codons "left over". Most amino acids are coded for by more than a single unique triplet, and therefore the genetic code is said to be **degenerate**.

Experiments which led to the solution of the genetic code:

Nirenberg and Matthei (1961): Nirenberg and Matthei worked with bacterial extracts which contained everything needed for translation, with the exception of mRNA. To this they added either poly A, poly U or poly C RNA. The proteins produced by the translation of these RNA's was determined (*poly G did not work, probably due to conformational problems*):

Poly U	Poly A	Poly C
Phe	**Lys**	**Pro**

Thus, the triplet UUU = Phe, AAA = Lys, and CCC = Pro.

Korana (1963): In a cell free extract system, Korana added mRNA with repeating nucleotide sequences. The sequence ...ACACACAC... resulted in a polypeptide with alternating **threonine and histidine** residues. But, was threonine coded by ACA, and histidine by CAC? Or vise versa? *To determine the answer to this, the mRNA sequence ...AACAACAACAAC... was tried.* There were three different possible reading frames for the translation of this mRNA:

- AAC AAC AAC
- ACA ACA ACA
- CAA CAA CAA

But CAC was not a possible triplet. This sequence was found to code for three different polypeptide chains: poly Asn, poly Thr, and poly Gln. Since no histidine was found, histidine was therefore coded for by the triplet CAC.

Nirenberg and Leder (1964): Nirenberg and Leder used a filter which would allow RNA triplets and charged tRNA's to pass through, but would prevent passage of larger ribosomes. Specific triplet RNA sequences would bind to ribosomes and cause the binding of the associated charged tRNA molecules (coded for by the specific triplet). In a given experiment, if a unique charged tRNA were radiolabeled (on the amino acid), then it could be determined whether that particular charged tRNA was associated for by the unique triplet. In this way, all 61 codons for amino acids were determined.

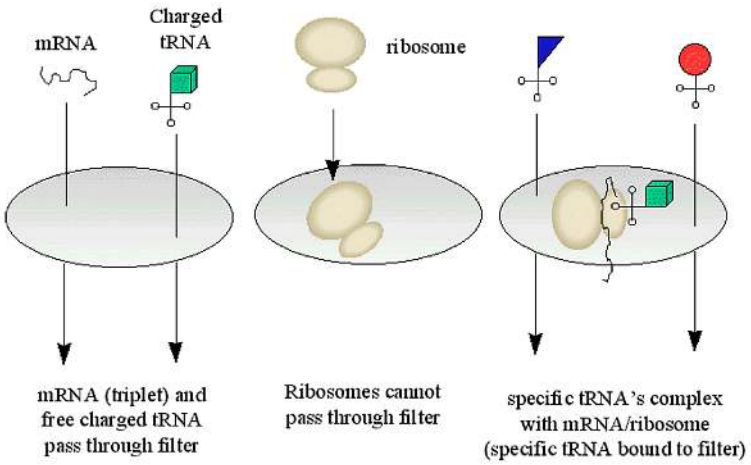


Figure 2.4.3: *Nirenberg and Leder experiment*

The genetic code

5' End (Start)	Second Position				3' End
	U	**C**	**A**	**G**	
U	Phe 0.24	Ser 0.34	Tyr 0.25	Cys 0.49	**U**
	Phe 0.76	Ser 0.37	Tyr 0.75	Cys 0.51	**C**
	Leu 0.02	Ser 0.02	**Stop**	**Stop**	**A**
	Leu 0.03	Ser 0.04	**Stop**	Trp 1.00	**G**
C	Leu 0.04	Pro 0.08	His 0.17	Arg 0.74	**U**
	Leu 0.07	Pro 0.00	His 0.83	Arg 0.25	**C**
	Leu 0.00	Pro 0.15	Gln 0.14	Arg 0.01	**A**
	Leu 0.83	Pro 0.77	Gln 0.86	Arg 0.00	**G**
A	Ile 0.17	Thr 0.35	Asn 0.06	Ser 0.03	**U**
	Ile 0.83	Thr 0.55	Asn 0.94	Ser 0.20	**C**
	Ile 0.00	Thr 0.04	Lys 0.74	Arg 0.00	**A**
	Met (start) 1.00	Thr 0.07	Lys 0.26	Arg 0.00	**G**
G	Val 0.51	Ala 0.35	Asp 0.33	Gly 0.59	**U**
	Val 0.07	Ala 0.10	Asp 0.67	Gly 0.38	**C**
	Val 0.26	Ala 0.28	Glu 0.78	Gly 0.00	**A**
	Val 0.16	Ala 0.26	Glu 0.22	Gly 0.02	**G**

Note

E. coli codon preferences are indicated.

All proteins in prokaryotes and eukaryotes begin translation with the *initiator* codon AUG (methionine). The three codons, UAA, UGA and UAG are **termination codons** (don't code for any amino acids but signal the end of the protein chain).

Note the apparent relative importance of the middle base in the codon triplet

Middle base in codon triplet

U	C	A	G
Phe Leu Ile Met Val	Ser Pro Thr Ala	Tyr His Gln Asn Lys Asp Glu Stop	Cys Trp Arg Ser Gly Stop
Hydrophobic	*Small/Polar*	*Charged/Polar*	*Polar*

Can common protein architectures be patterned by a simple quaternary pattern of residues?

The twenty common amino acids and their three-letter and single-letter acronyms:

Amino Acid	Three letter acronym	One letter acronym
Alanine	Ala	A
Cysteine	Cys	C
Aspartic Acid	Asp	D
Glutamic Acid	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Tyr	Y

This page titled 10.2.4: Central Dogma and the Genetic Code is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **2.4: Central Dogma and the Genetic Code** by Michael Blaber has no license indicated.

10.2.5: Gene and Operon

Genes

The entire nucleic acid sequence that is necessary for the synthesis of a functional polypeptide or RNA molecule. Thus, a genecontains additional sequence information beyond that which codes for the amino acids in a protein or the nucleotides in an RNA molecule. The gene also contains the DNA necessary to get a particular transcript made.

Transcription control regions can be remote to the coding region (on the order of Kb's or 10's of Kb's away).

Most prokaryotic genes lack introns (intervening DNA sequence). In prokaryotes, genes which encode proteins with relationships in a metabolic pathway form *Operons* - which produce *polycistronic mRNA's*.

Definitions: Operon and Promoter

- An operon is in bacterial DNA, a cluster of contiguous genes transcribed from one promoter that gives rise to a polycistronic mRNA.
- A Promoter is a DNA sequence to which RNA polymerase binds prior to initiation of transcription - usually found just upstream of the transcription start site of a gene

e.g. *Trp* Operon - involved in the biosynthesis of the amino acid tryptophan:

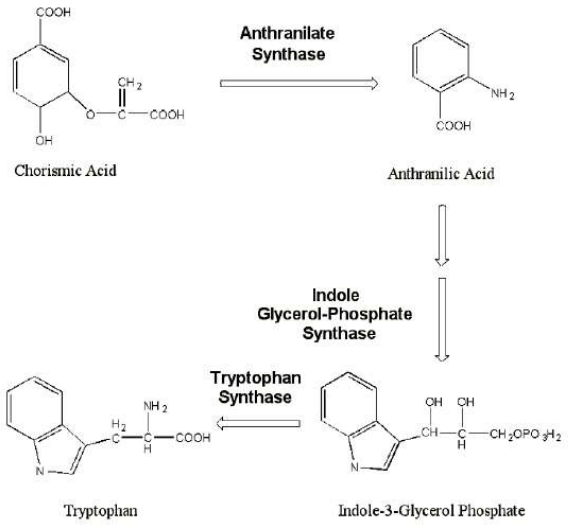


Figure 2.5.1: *Chemical pathway of trp operon*

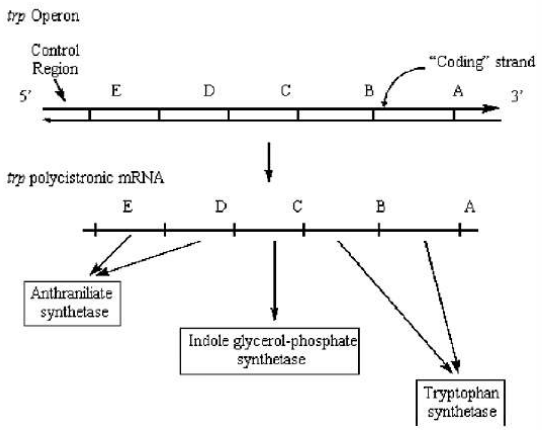


Figure 2.5.2: *Trp operon in DNA/RNA*

A consequence of the arrangement of bacteria genes into operons is that *the level of mRNA for each of the genes in the operon is exactly the same*.

> Ribosomes transcribe from the start of each gene, not only from the first gene.

Another consequence of the arrangement of bacteria genes into operons is that an upstream mutation (i.e. possibly inhibiting transcription) can prevent "downstream" genes from being transcribed and expressed. Most eukaryotic transcription units produce *monocistronic* mRNA's, (i.e. they encode only one protein). There is a fundamental difference in the translation processes of prokaryotes and eukaryotes:

1. In prokaryotes ribosomes can bind at specific recognition sequences anywhere within the mRNA (called ribosome binding sites, or "Shine-Dalgarno" sites).
2. In eukaryotes, ribosomes bind via the interaction with specifically modified 5' region (so called ***5' cap site***) of mRNA molecules.
3. Most eukaryotic mRNA's are therefore ***monocistronic***.

- Mutations in simple eukaryotic transcription units affect only one protein.

Complex Eukaryotic Transcription Units

The primary RNA transcript encoded by complex transcription units can be *spliced*in more than one way. Because of the different processing possibilities, the *exons* (coding regions) in a single complex transcription unit can be linked in alternative ways, to yield *different mRNAs* and *different proteins*.

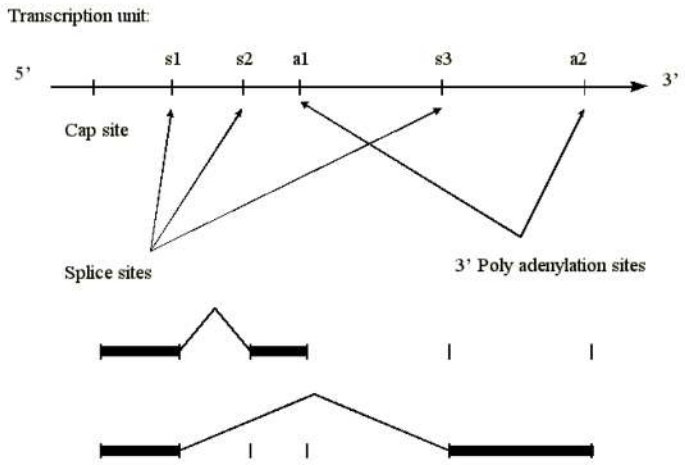


Figure 2.5.3: *Complex transcription units*

Transcriptional regulation

Successful survival requires adaptability and economy:

1. The ability to switch from metabolizing one substrate to another as environmental resources change
2. It would be an energetic waste to produce enzymes for a metabolic pathway which is not needed.

Induction versus Repression of Enzyme Synthesis

In *E. coli* certain enzymes are produced only when the cells are grown on certain substrates. This effect is called enzyme *induction*. For example, when cells are grown in the absence of a type of sugar known as a *b galactoside* (e.g. lactose) the cells contain very few molecules (~5 per cell) of the enzyme *b-galactosidase* (which cleaves lactose into glucose and galactose).

- There is no need for this enzyme in the absence of lactose.
- If lactose is added to *E. coli*, in a very short amount of time there are approximately **5000 molecules** of b-galactosidase per cell (approximately ~1,000 fold induction).

- If lactose is removed from the media **synthesis** of b-galactosidase **stops**.

A similar but opposite situation occurs in regard to the synthesis of tryptophan (the biosynthetic enzymes are contained in the ***trp* operon**). In this case production of the enzymes for tryptophan biosynthesis are rapidly **shut down** if tryptophan is **present**, in a process called *repression*. Repression is a transcriptional regulatory mechanism for commonly required gene products. Induction is a transcriptional regulatory mechanism for gene products which may be required under unusual or infrequent situations.

This page titled 10.2.5: Gene and Operon is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **2.5: Gene and Operon** by Michael Blaber has no license indicated.

10.2.6: The lac Operon; CAP site; DNA footprinting

The *E. coli* lac operon

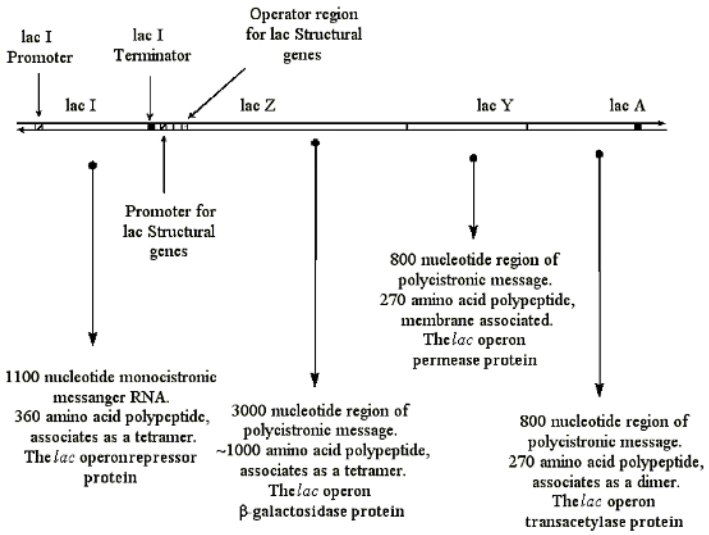


Figure 2.6.1: *lac Operon*

- ***lac* Z** codes for **b-galactosidase**, which is an enzyme that cleaves b-galactosides (e.g. lactose).
- ***lac* Y** codes for **permease**, which is involved in the transport of b-galactosides into the cell.
- ***lac* A** codes for **b-galactoside transacetylase**, which acetylates b-galactosides.

- A mutation in either *lac* Z or *lac* Y can lead to a *lac*$^-$ genotype, i.e. *cells which cannot utilize b-galactosides as a nutrient.*
- A *lac* A- mutant, lacking transacetylase activity, can still utilize b-galactosides (it is still lac+ genotype). Its role in the metabolism of bgalactosides is not clear.

Promoter

a region of DNA involved in binding of RNA polymerase to initiate transcription.

Terminator

a sequence of DNA that causes RNA polymerase to terminate transcription.

- The cluster of three genes, ***lac* ZYA**, is transcribed into a single mRNA (***polycistronic*** message) from a **promoter** just upstream from the *lac* Z gene.
- In the *absence* of an inducer the gene cluster is *not*transcribed.
- When an inducer is added (e.g. lactose, or the non-hydrolyzable analog isopropyl thiogalactoside - IPTG) transcription starts at a single promoter (***lac* P**) and proceeds through the lac ZYA genes to a terminator sequence located downstream of the **lac A** gene.

Note

The lac ZYA mRNA has a half life of ~3 minutes, which allows induction to be reversed relatively rapidly (i.e. cells stop producing enzymes rapidly after induction stops).

Exercise 2.6.1

What molecule does the inducer (lactose) interact with to affect transcriptional regulation (i.e. induction of the lac operon)?

Answer

It is not b-galactosidase, permase or transacetylase, rather it is a separate protein called a repressor protein.

- The lac genes are controlled by a mechanism called **negative regulation**.
  - This means that *they are transcribed unless they are turned off by the regulator protein.*
  - A mutation that **inactivates the regulator protein** causes the *lac*ZYA genes to be **continually expressed**.

Since the function of the regulator protein is to prevent expression, it is called a repressor protein.

There are two types of genes in the lac operon:

1. ***Structural genes*** - they code for enzymes required for some biochemical pathway (e.g. lac Z, Y and A).
2. ***Regulator genes*** - they code for proteins involved in regulation of structural genes.

*Mutations in **structural** genes typically affect the function of only that structural gene.*
*Mutations in **regulator** genes can affect the expression of all structural genes in an operon.*

***lac* I is the regulator gene of the lac operon.**

- This gene is located just upstream of the promoter region for the lac structural genes.
- The *lac* I gene has its own *promoter* (***constitutive***) and terminator.
- It makes a monocistronic message, and codes for one protein - the *lac* repressor protein.

The crucial feature of the lac "control circuit" resides in the dual feature of the lac I repressor protein:

1. It can prevent transcription
2. It can recognize and bind the small molecule inducer (lactose or IPTG)

Prevention of transcription by the lac repressor

- lac repressor (active as a ***tetrameric*** protein) binds to a sequence of DNA called the ***operator*** (*lac* O region).
  - The operator region lies between the *lac* promoter region (site of RNA polymerase binding and transcription initiation) and the *lac* Z gene.
  - The first 26 base pairs of the *lac* Z gene comprise the operator region.

When the repressor binds to the operator region, its presence prevents RNA polymerase from initiating transcription at the promoter.

- It is not that the repressor protein "blocks" the movement of RNA polymerase through the *lac* Z gene.
- Repressor binding and RNA polymerase binding (to the promoter) are mutually exclusive at the *lac* promoter/operator (*lac* PO) region.

How does the repressor/operator interaction change in the presence of the inducer molecule?

- The ***inducer*** can bind to the repressor to form a *repressor/inducer complex that no longer associates with the operator*.
  - The key feature of this interaction is that *the repressor protein has two binding sites*, one for the inducer and one for the operator.
  - When the inducer binds at its site, it changes the conformation of the repressor protein such that the operator binding site has a much reduced affinity for the DNA operator region.
  - This type of control is called *allosteric control*.
  - The result is that *when the inducer is added the repressor is converted to a form which releases from the operator*.

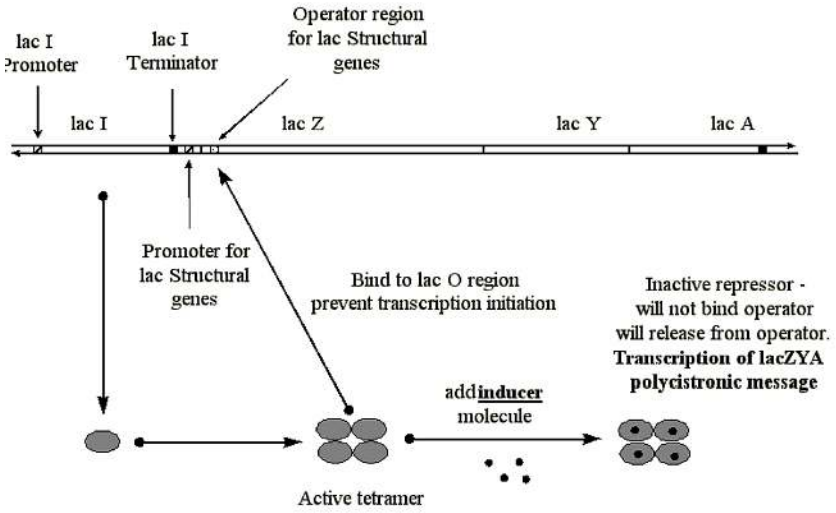


Figure 2.6.2: *Inducer*

Positive control of the lac operon is exerted by cAMP-CAP complex

- *E. coli* prefers **glucose** over other carbon sources.

  When glucose enters an E. coli cell it is utilized directly without induction of any new enzymes.

- When *E. coli* is grown on glucose, if another sugar (e.g. lactose) is added *the induction of enzymes to utilize the other sugar does not occur until the glucose is used up.*

- When E. coli is starved for glucose it synthesizes an unusual nucleotide: *cyclic 3'5' adenosine monophosphate* (cyclic AMP, or *cAMP*):

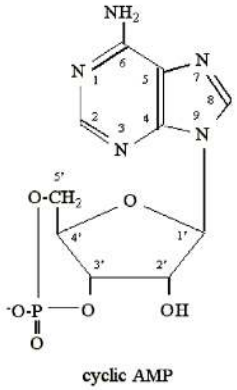


Figure 2.6.3: *cAMP*

1. In bacteria an increase in the cAMP level seems to be an "alert" signal indicating a low glucose level:

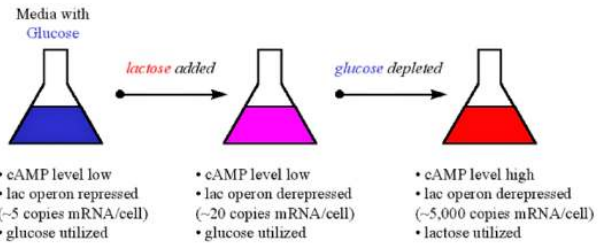


Figure 2.6.4: *Interaction of cAMP level and lac operon*

Dibutyryl cAMP

- an *analogue* of cAMP which can pass through the *E. coli* membrane and into the cell.
- If this is added to media containing glucose and lactose it will result in the **induction** of the *lac* operon.
- Thus, it mimics the chemical message which tricks the *E. coli* to respond as though glucose levels were low.
- Mutants of *E. coli* have been isolated which cannot be induced to metabolize ***any*** sugar other than glucose. There were two general categories of mutants:

1. Class I. *Defective in the enzyme* ***adenylate cyclase***. These mutants are unable to make cAMP even when the glucose conentration is low.
2. Class II. Lacks a particular protein known as ***cAMP receptor protein*** (CRP) or, also known as catabolite receptor protein (CRP).

- Maximum transcription from the *lac* operon requires the presence of a ***cAMP/CRP*** complex.
  - cAMP/CRP complex binds to a specific sequence in the lac control region called the "**CAP**" site.
  - The CAP site is just ***upstream*** from the RNA polymerase binding site.
  - Mutations in the CAP site that prevent cAMP-CRP binding also prevent high levels of expression of the *lac* operon.
- Thus, bound cAMP/CRP complex ***activates*** transcription (**positive control**), whereas bound *lac* repressor ***inhibits*** transcription (**negative control**).
  - cAMP/CRP complex has affinity for DNA, and RNA pol.
  - Enhances complex formation of RNA pol with the DNA promoter region.

Induction of the *lac* operon with lactose analogues

- The *lac* operon can be induced with *lactose*
  - b-galactosidase (*lac*Z gene product) metabolizes the lactose
  - When levels of lactose are reduced, the *lac* operon is again repressed by the *lac* repressor (*lac*I gene product)
- *Non-metabolized lactose analogues* can continually induce (i.e. de-repress) the *lac* operon
  - isopropyl b-thiogalactoside, or **IPTG**, is a non-metabolized lactose analogue

DNA "footprinting" experiments

- If a protein binds to a region of DNA, *it can protect that region of DNA from digestion by dnase* (**DNAse I**: an endonuclease at sites adjacent to pyrimidine nucleotides).
  - A fragment of DNA can be labeled at the 5' ends with ^{32}P and then the label can be preferentially removed from one end (i.e. the 3' end of a gene) by an appropriate restriction endonuclease.
  - If this DNA fragment, with a label at one specific end, forms a complex with a DNA binding protein the protein will protect the region of DNA that it binds to from DNAse I digestion.
  - The digestion is done so as to be incomplete, for the purposes of this discussion, imagine that each DNA molecule is cleaved only once. Furthermore, the site of cleavage is randomly chosen from the available sites.
  - Fragments of the DNA, separated and analyzed by size (using gel electrophoresis) after digestion will indicate the protected region:

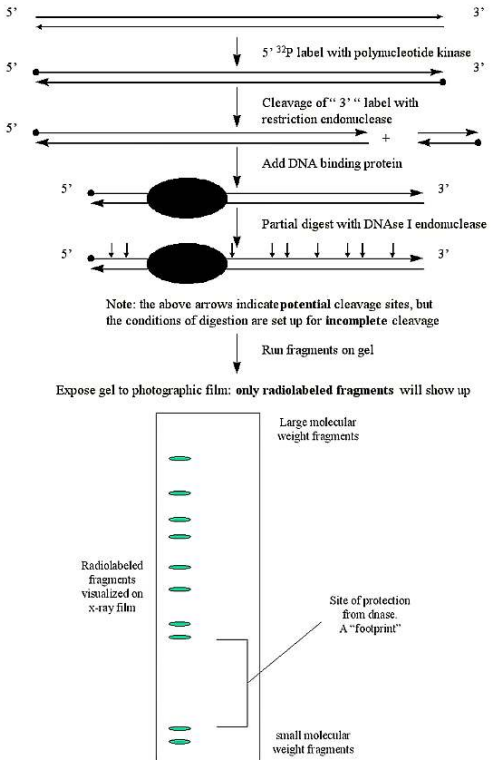


Figure 2.6.5: *DNA footprinting*

Results of footprinting experiments

lac DNA incubated with either cAMP/CAP protein, or RNA polymerase, or lac I repressor protein:

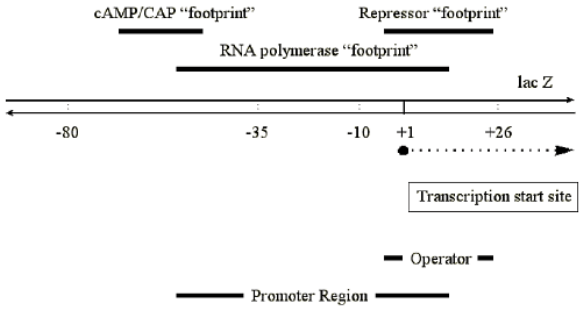


Figure 2.6.6: *lac repressor with cAMP*

RNA polymerase interacts with specific promoter sequences and produces a "footprint" over a region of ~70 base pairs.

- This protection was observed to be more obvious on one strand than the other (i.e. if the other strand was labeled the results did not show as much protection).
- This region of DNAse protection included sites in the DNA from which mutagenesis experiments produced either "up" regulation or "down" regulation of promoter strength.
- These mutagenic "hot" spots affecting promoter strength were located at positions either -10 or -30 upstream from the transcription start site (position +1 in the above diagram):

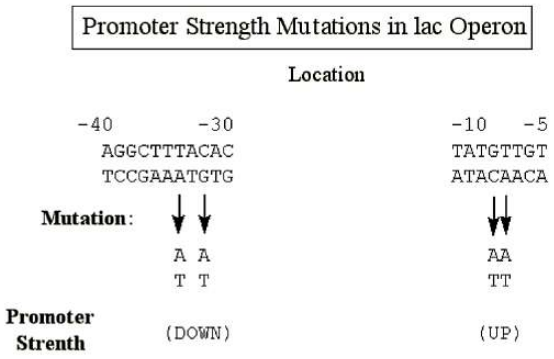


Figure 2.6.7: *Promoter Strength Mutations*

- Promoters can be classified according to their "strength".
- This refers to the relative ***frequency of transcription initiation*** (transcriptional initiation events per minute), and is related to the ***affinity of RNA polymerase for the promoter region***.
- Many promoters in *E. coli* have been characterized and a "consensus" promoter sequence has been identified:

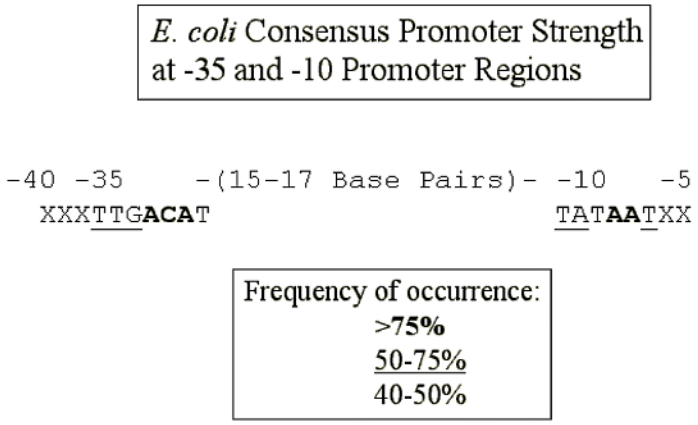


Figure 2.6.8: *Consensus Promoter Strength*

> **Note**
>
> The lac promoter is a relatively weak promoter

RNA Polymerase

- *E. coli* RNA polymerase is a holoenzyme comprised of subunits b', b, a (dimer) and s^{70}.
  - The s^{70} subunit is the subunit which binds to the promoter region, but is unable to initiate RNA synthesis.
  - After the s^{70} subunit subunit binds, the other subunits bind forming a function RNA polymerase.
  - After approximately 10 base pairs have been transcribed the s^{70} subunit leaves and the **core polymerase** continues on.

The *lac* Operator Region

- The *lac* operator region is comprised of an (imperfect) ***inverted repeat*** region.
- Not surprisingly active repressor molecules are composed of a homodimer.
  - In the homodimer structure there are a pair of a-helix regions which insert into adjacent major grooves of the DNA.
  - The separation is approximately 34 angstroms apart.

This page titled 10.2.6: The lac Operon; CAP site; DNA footprinting is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **2.6: The lac Operon; CAP site; DNA footprinting** by Michael Blaber has no license indicated.

10.2.7: Transcriptional Regulation- Transcription termination, the trp operon

Transcription termination in prokaryotes

- Because protein coding regions are closely spaced in the genomes of microorganisms, independent control of neighboring genes is possible only if a **transcription termination site** lies between them (i.e. downstream genes must be independently regulated).
- There are two general mechanisms of transcription termination:

1. one requires the presence of a transcription termination protein called ***rho***,
2. the other requires no associated proteins.

Rho-independent termination

- *Rho*-independent transcription termination sequences have two general characteristic features:

1. A 3' stretch of T residues
2. A 'GC' rich interrupted palindrome just upstream of the 3' poly T region.

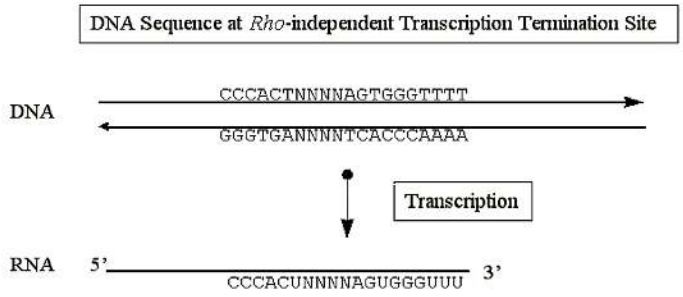


Figure 2.7.1: *Rho-independent transcription termination site*

The important features of Rho-independent transcription termination are as follows:

1. The inverted repeat region can self-hybridize to form a "stem-loop" structure:

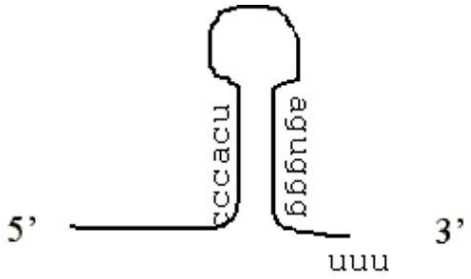


Figure 2.7.2: *Stem-loop structure*

2. The GC rich stem loop structure interacts with RNA polymerase to cause it to pause.

3. The short UUU region, which is base pairing with AAA sequence on the anti-sense DNA strand, has low thermal stability and melts - releasing the nascent RNA transcript.

Attenuation at the trp operon and premature mRNA chain termination

- The *trp* operon is regulated by the *trp* repressor protein.
  - *Trp* repressor protein binds to tryptophan and undergoes a conformational change which allows it to bind to the *trp* operator region (downstream of the promoter).
  - This prevents transcription of the *trp* operon in the **presence** of tryptophan (i.e. tryptophan represses expression).
  - However the repression is somewhat incomplete and **there is a second mechanism which contributes to transcription repression**.

- When tryptophan is present, and the operon is repressed, the few transcripts which are made are actually **quite short** and do not encompass the entire polycistronic message of the *trp* operon.
- The short mRNA that is made comprises only a region called the leader sequence:

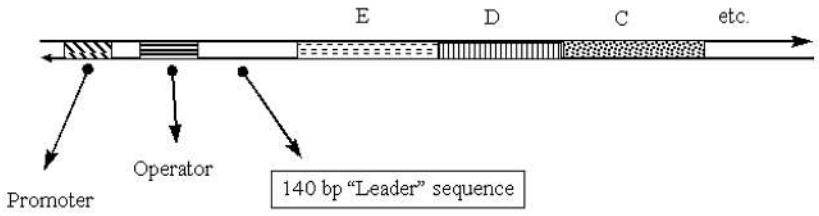


Figure 2.7.3: *Leader sequence*

- The leader region contains an attenuator sequence - a site where a choice is made between **elongation** of the growing *trp* transcript or (premature) **termination**.
- ***Attenuation*** depends on the interplay between *ribosome binding* and translation of the nascent mRNA transcript and the formation of a particular *stem-loop structure* in the mRNA leader sequence.
  - Formation of this structure depends on the rate of ribosomal translation of the leader sequence (**the leader sequence contains an AUG start codon**).
  - *Efficient translation of the leader sequence depends upon the concentration of charged tRNA's for the appropriate amino acids coded for by the mRNA leader sequence*.
- The leader sequence has the following characteristics:

1. It contains a ribosome binding site and an AUG start codon necessary for the initiation of translation.
2. The corresponding amino acid sequence coded for by the leader sequence contains several **tryptophan residues**.
3. It contains several **inverted repeat sequences** such that the mRNA can adopt *different alternative structures*.
4. One of these structures represents a *rho*-independent **termination site**.

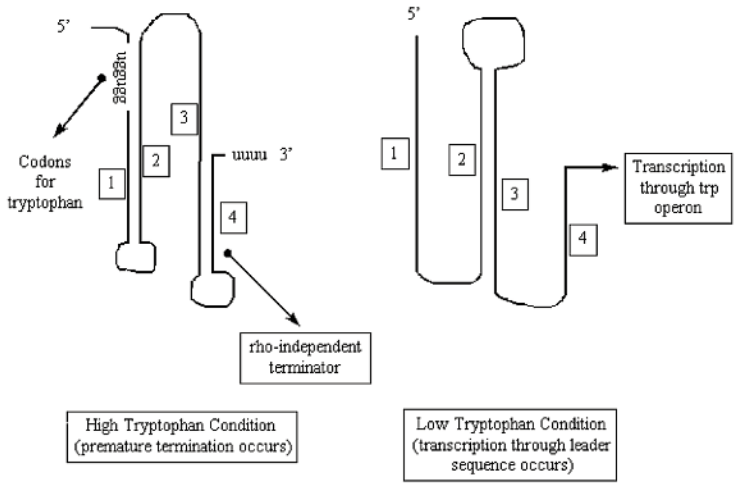


Figure 2.7.4: *High vs. Low tryptophan*

Under conditions of low tryptophan

- A **ribosome** translating the nascent *trp* polycistronic mRNA **stalls** at the codons for tryptophan in the leader mRNA sequence.
  - This **prevents** segments 1 and 2 of the nascent mRNA from forming a potential stem-loop structure, and frees up segment 2 to form a stem loop structure with segment 3.

- In this case, nascent transcription can proceed through, transcribing the entire *trp* operon.

Under conditions of high tryptophan

- A **ribosome** translating the nascent *trp* polycistronic mRNA does **not** stall at segment 1 (the region with codons coding for tryptophan).
  - In this case the stem-loop structure between 1 and 2 is **not prevented from forming**, and thus the stem-loop structure **3-4 can form**.
  - This stem loop structure is essentially a ***rho*-independent transcription termination structure**.
  - The RNA polymerase transcribing the nascent message will terminate.

Rho-dependent transcription termination

- About half the termination sites in *E. coli* require an accessory protein called the **rho factor**.
- Many *rho*-dependent sites have been characterized from *E. coli* and **no obvious sequence similarity is present**.
- Rho associates with the nascent RNA transcript and this interaction activates an ATPase activity which appears to allow *rho* to **translocate** along the mRNA in the **3' direction**.
  - It may be that if the RNA polymerase **pauses**, *rho* can catch up and cause termination (i.e. bump RNA polymerase off the DNA template?).
  - Pausing of RNA polymerase is thought to be an important mechanism of rho-dependent transcription termination.

This page titled 10.2.7: Transcriptional Regulation- Transcription termination, the trp operon is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **2.7: Transcriptional Regulation: Transcription termination, the trp operon** by Michael Blaber has no license indicated.

10.2.8: mRNA Translation

How is the code contained in mRNA translated into a protein?

Structure and function of transfer RNA's

- **tRNA's** have ***two*** functions:
  1. To chemically link to a particular amino acid (***covalent***)
  2. To recognize a specific **codon in mRNA** (***non-covalent***) so that its attached amino acid can be added to a growing peptide chain

Amino-acyl tRNA synthetases

- Function is to ***"charge"*** tRNA molecules; i.e. *to chemically link a specific amino acid to its associated tRNA molecule*.

Amino Acids	Amino-acyl tRNA synthetases	tRNA's	Codons
20	20	30-40 (prokaryotes) 50 (eukaryotes)	61 (3 stop codons)

Conclusions:

1. *There is one amino-acyl tRNA synthetase per amino acid* (they are quite specific).
2. *There is potentially more than one tRNA per amino acid*.

Therefore, amino-acyl tRNA synthetases must be able to recognize more than one tRNA.

1. *There is potentially more than one codon per tRNA.*

Therefore each tRNA must be able to recognize more than one codon (there is not a unique tRNA for each codon).

Structure of tRNA's

- 70-80 nucleotides long
- Form a series of stem/loop secondary structures
- tRNA's are synthesized with the standard bases AGCU. However, after synthesis several bases may be modified:
  1. Uridylate may be **methylated** to produce **Thymidylate**
  2. Uridylate may be **rearranged** to produce **pseudouridylate** (i.e. ribose attached to Carbon 5 instead of Nitrogen 1).
  3. Guanidylate may be **methylated** at different positions.
- The amino acid is attached at the 3' end of the tRNA to either the 2' hydroxyl or the 3' hydroxyl.
  1. ***Class I*** *amino-acyl tRNA synthetases attach their associated amino acids to the tRNA* ***2' hydroxyl*** (NOTE: typically the hydrophobic amino acids)
  2. ***Class II*** *amino-acyl tRNA synthetases attach their associated amino acids to the tRNA* ***3' hydroxyl*** (NOTE: typically hydrophilic amino acids)

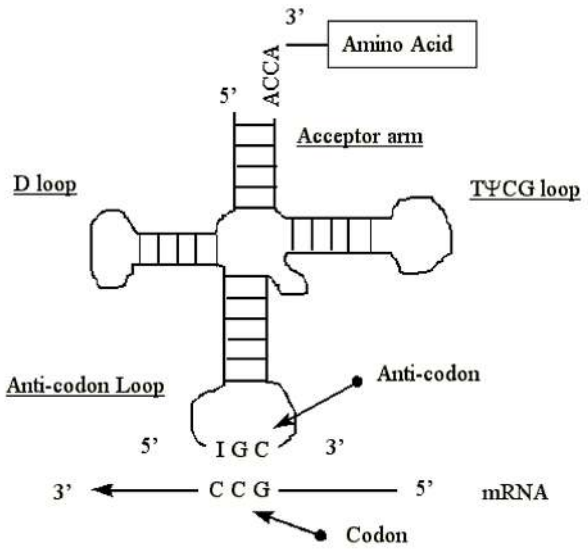


Figure 2.8.1: *tRNA*

- If perfect Watson-Crick base pairing were required at the codon/anti-codon triplet then ***61 different tRNA's would be required***.
- We know this is not the case, therefore a single tRNA anti-codon must be able to recognized several different mRNA codon triplets.
- This greater recognition of tRNA is possible due to "**wobble**" basepair interactions at the third base in the codon/first base in the anti-codon:

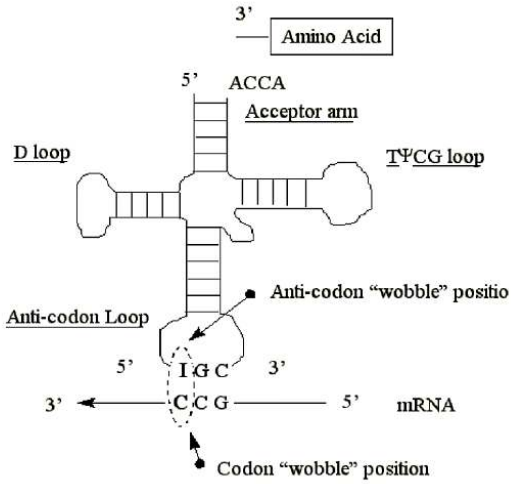


Figure 2.8.2: *Codon wobble*

Possible "wobble" codon base pairing (in addition to Watson-Crick):

1. **U - G**
2. **I - C**
3. **I - A**
4. **I - U**

- Where U, G, A and C can be in ***either*** the codon (mRNA) or anti-codon (tRNA)
- **I** (inosine) can be found in the ***anti-codon***.

For example, the codons UUU and UUC are both recognized by the tRNA which has GAA in the anti-codon position (making either G - C, or G - U base pairings).

Recognition of amino acids by amino-acyl tRNA synthetases

- Appears to involve not only the anti-codon triplet but significant other contacts as well (mostly involving the acceptor stem region).

Ribosomes

- The mRNA with its encoded information and the individual tRNAs loaded with their amino acids are brought together by a mutual affinity for an RNA-protein complex called the **Ribosome**.
- The rate of protein synthesis by a ribosome is approximately ***3-5 amino acids/minute***.
  - For example, a large protein (e.g. Titin, 30,000 amino acids) takes 2-3 hours to make.
- Ribosomes are composed of individual ribosomal RNA (rRNA) molecules and more than 50 accessory proteins, with a general prokaryotic organization of a **small subunit (30S)** and a **large subunit (50S)**.

Translation of mRNA to proteins

- Protein synthesis is usually considered in three steps:

1. *Initiation*
2. *Elongation*
3. *Termination*

AUG is the initiation signal in mRNA

- The **first** event of the initiation stage is the attachment of a free molecule of methionine (Met) to the end of a $tRNA^{Met}$ by a specific aminoacyl-tRNA synthetase.
- There are at least **two** types of $tRNA^{Met}$:
  1. $tRNA_i^{Met}$: can **initiate** protein synthesis (at **AUG** met codon)
  2. $tRNA^{Met}$: can **incorporate** Met residues during on-going protein synthesis (at **AUG** met codon)
- Methionine tRNA synthetase attaches Methionine to both tRNA molecules.
- Only methionyl-$tRNA_i^{Met}$ can bind to the small ribosomal subunit to begin the process of protein synthesis.
  - In bacteria, the amino group of the methionine in methionyl$tRNA_i^{Met}$ is ***formylated***.
  - The Met-$tRNA_i^{Met}$, along with a protein-GTP complex and the small (30S) ribosomal subunit bind to the mRNA at a specific site, **near** the AUG initiation codon.

Initiation of protein synthesis

- In most prokaryotes an RNA component (16S rRNA) in the small rRNA subunit (30S) recognizes and hybridizes to a specific sequence on the mRNA called **the Shine-Dalgarno** sequence:

mRNA 5' -**UAAGGAGG** -(5-10 nucleotides)-**AUG** 3'
16S rRNA OH-**AUUCCUCC** -(~1400 nucleotides)-5'

- The Shine-Dalgarno sequence is thus a **ribosome binding site** which is necessary for the intiation of translation.
  - Note that the ribosome does not bind at the AUG start codon, but 5-10 nucleotides **upstream**.
  - The Shine-Dalgarno sequence can be located **anywhere** within an mRNA.
- A series of **initiation factors**, **Met-$tRNA_i^{Met}$** , **mRNA** and the **30S (i.e. 16S component)** ribosomal subunit are necessary for formation of the **30S initiation complex**.
- The large (50S rRNA) rRNA binds along with release of initiation factors 1 and 2, and hydrolysis of GTP, to form the 70S inititation complex:

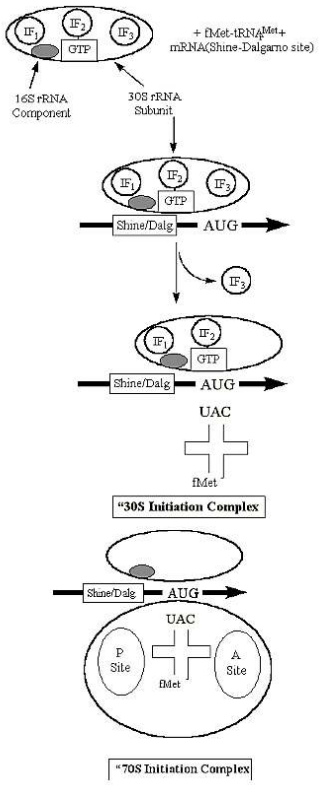


Figure 2.8.3: *Initiation complexes*

Elongation

1. In the first part of the **elongation step**of translation, the ribosome moves along the mRNA to position the fMet residue to the **P site (peptidyl site)** in the 50S subunit.
   - This allows the **second codon** of the mRNA to be positioned in the **A site (amino acyl tRNA site)**.
2. The appropriate charged tRNA (with amino acid) specified by the second codon is positioned in the **A site** of the 50S subunit.
3. Next peptide bond formation is synthesized and the tRNA in the A site (which is covalently attached to the nascent polypeptide) is translocated to the **P site**.
   - This process requires **GTP and the G elongation factor** protein (prokaryotes).
4. The process is repeated.

Termination

1. When a stop codon is reached the **polypeptide** is hydrolyzed away from the last tRNA.
   - The peptide is released and the ribosome typically dissociates.
   - This process requires GTP and three different termination factors (TF's; only one required in Eukaryotes)

This page titled 10.2.8: mRNA Translation is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **2.8: mRNA Translation** by Michael Blaber has no license indicated.

SECTION OVERVIEW

10.3: 3. Biotechnology 1

10.3.1: Gel Electrophoresis

10.3.2: DNA Sequence Analysis

10.3.3: Polymerase Chain Reaction (PCR) and Cloning of PCR Products

10.3.4: Prokaryotic Expression Vectors

10.3.5: Protein Sequencing, Peptide Mapping, Synthetic Genes

10.3.6: cDNA and Genomic Libraries

10.3.7: Protein Purification

This page titled 10.3: 3. Biotechnology 1 is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

10.3.1: Gel Electrophoresis

Gel electrophoresis is used to characterize one of the most basic properties - molecular mass - of both polynucleotides and polypeptides. Gel electrophoresis can also be used to determine: (1) the **purity** of these samples, (2) **heterogeneity/extent** of degradation, and (3) subunit **composition**.

DNA

The most common gel electrophoresis materials for DNA molecules is **agarose** and **acrylamide.**

DNA agarose gels

The electrophoretic migration rate of DNA through agarose gels is dependent upon ***four main parameters***:

1. The **molecular size** of the DNA. Molecules of linear duplex DNA travel through agarose gels at a rate which is inversely proportional to the log of their molecular weight.

$$M_r \propto 1/log(Mw) \tag{10.3.1.1}$$

Example: Compare molecular mass vs. expected migration rate:

Molecular Mass (Da)	log (Molec. Mass)	1/log (Molec. Mass) i.e. relative Mr
100,000	5.0	0.20
50,000	4.7	0.21
10,000	4.0	0.25
5,000	3.7	0.27
1,000	3.0	0.33

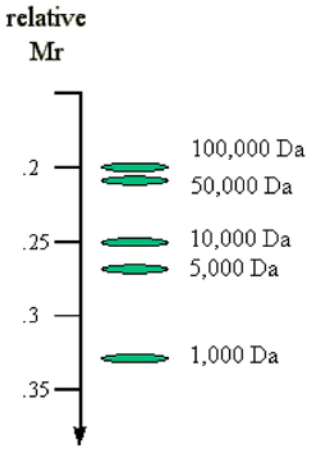


Figure 3.1.1: *Relative migration rate with molecular mass*

2. The **agarose concentration**. There is an inverse linear relationship between the logarithm of the electrophoretic mobility and gel concentration.

$$\log(M_r) \propto 1/[gel] \tag{10.3.1.2}$$

Gel %	1/Gel %	inv log(1/Gel %) (i.e. relative Mr)
2.0	0.50	3.2
1.5	0.67	4.6
1.0	1.00	10.0
0.5	2.00	100.0

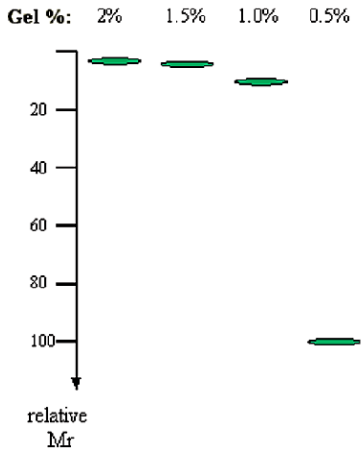


Figure 3.1.2: *Relative migration rate with gel concentration*

3. The conformation of the DNA.

- closed circular DNA (***form-I***) - typically supercoiled
- nicked circular (***form-II***)
- linear DNA (***form-III***)

These different forms of the *same DNA* migrate at different rates through an agarose gel. Almost always the **linear form** (form-III) migrates at the **slowest** rate of the three forms and supercoiled DNA (form-I) usually migrates the **fastest.**

4. The applied **voltage**.

- Typical value for running an agarose gel is **5 volts per cm** (length of gel).

Agarose (%)	Range of separation of linear DNA (in kilobases)
0.3	60 - 5
0.6	20 - 1
0.7	10 - 0.8
0.9	7 - 0.5
1.2	6 - 0.4
1.5	4 - 0.2
2.0	3 - 0.1

- Agarose gels are usually poured and run horizontally

Finally, the DNA being an acidic molecule, migrates towards the positively charged electrode (cathode).

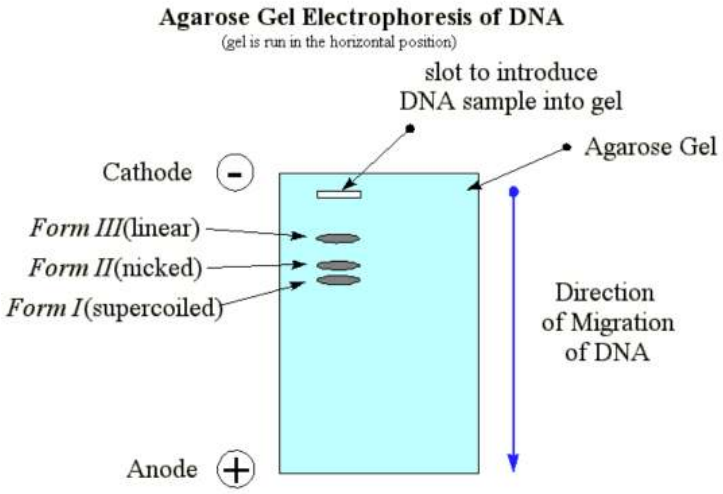


Figure 3.1.3: *Gel electrophoresis setup*

DNA acrylamide Gels

Acrylamide gels are useful for separation of **small DNA fragments** typically oligonucleotides <100 base pairs. These gels are usually of a *low acrylamide concentration* (<=6%) and contain the non-ionic *denaturing agent* Urea (6M). The denaturing agent prevents secondary structure formation in oligonucleotides and allows a relatively accurate determination of molecular mass.

Gel Electrophoresis for Proteins

Gel electrophoresis of proteins almost exclusively utilizes **polyacrylamide**. The acrylamide solution usually contains **two components**: acrylamide and bis acrylamide. A typical value for the acrylamide:bis ratio is 19:1. The bis acrylamide is essentially a **cross-linking** component of the acrylamide polymer. The ***total*** acrylamide concentration in the gel affects the migration of proteins through the matrix (as with the concentration of agarose).

Protein gels are usually performed under ***denaturing conditions*** in the presence of the detergent **sodium dodecyl sulfate (SDS)**. The proteins are denatured by heat in the presence of SDS. The SDS binds, via hydrophobic interactions, to the proteins in an amount *approximately proportional to the size of the protein*. Due to the charged nature of the SDS molecule the proteins thus have a somewhat ***constant charge to mass ratio*** and migrate through the gel at a rate proportional to their molecular mass, The proteins migrate towards the ***anode***.

Acrylamide (%)	Range of separation of Polypeptides (in kilodaltons)
8	200 - 25
10	100 - 15
12.5	70 - 10
15	60 - 6
20	40 - 4

Since the SDS treatment will dissociate non-covalent protein complexes, they may thus exhibit a much l*ower than expected* molecular mass on SDS polyacrylamide gel electrophoresis (**SDS PAGE**). Protein PAGE gels are usually polymerized between two glass plates and run in the vertical direction.

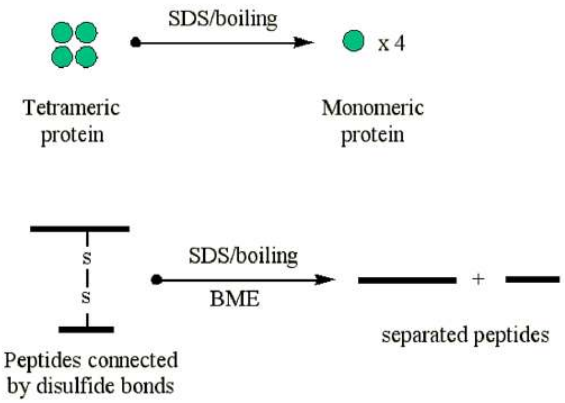


Figure 3.1.4: *Effect of SDS treatment*

PAGE may also be run in the presence of **reducing agents**, such as ***b-mercaptoethanol (BME)***. BME is a reducing agent which will reduce any disulfide bonds (e.g. as exists between some pairs of cysteine residues in a protein). This helps to remove residual secondary structure in the SDS treated protein, but it may also allow the separation of polypeptide fragments from each other (i.e. their covalent interaction was entirely made up of one or more disulfide bonds). Thus, an apparently single protein may exhibit a set of small fragments under reducing PAGE conditions.

Stains for DNA

Ethidium

The most convenient method to visualize DNA in gel electrophoresis is staining with the fluorescent dye **ethidium bromide**. This compound contains a planar group that **intercalates** between the stacked bases of DNA. The orientation and proximity of ethidium with the stacked bases causes the dye to display an increased florescence compared to free dye (in solution). U.V. radiation at 254 nm is absorbed by the DNA and transmitted to the bound dye and the energy is re-emitted at 590 nm in the **red-orange** region of the spectrum.

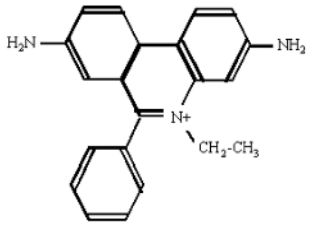


Figure 3.1.5: *Ethidium*

Ethidium bromide is usually prepared as a stock solution of 10 mg/ml in water, stored at room temp and protected from light. The dye is usually incorporated into the gel and running buffer, or conversely, the gel is stained after running by soaking in a solution of ethidium bromide (0.5 ug/ml for 30 min). The stain is visualized by irradiating with a UV light source (i.e. using a transiluminator) and photgraphing with polaroid film. The usual sensitivity of detection is better than 0.1 ug of DNA.

Because ethidium is a DNA intercalating agent, it is a powerful mutagen. Incorporation of ethidium in the DNA of living organisms (i.e. you and I) can cause (unwanted) mutations.

Note:

- The intercalation of ethidium bromide causes a reduction in the number of effective basepairs per twist.
- Thus, the DNA wants to adopt a conformation with fewer base pairs per twist.
  - The number of twists will increase for the given length of DNA
  - Writhe will decrease if the Linkage number remains unchanged
- The net result is that addition of ethidium bromide will introduce negative supercoils into the DNA

- *Addition of ethidium bromide to a DNA sample prior to electrophoresis can cause positively supercoiled DNA to migrate as relaxed (nicked, or form II) DNA*
- After replication DNA is typically *underwound* (negative supercoils), thus it will become *more* underwound with EtBr treatment (migration rate may actually *increase* due to more compact state)

Flourescence shadowing:

- DNA fragments resolved on polyacrylamide gels can also be visualized by the method of **UV shadowing**.
  - In this method the gel is placed on top of a fluorescent material, usually a flourescent TLC silica plate.
  - The gel is then illuminated by a UV light source.
  - DNA bands in the gel will block transmittance of the UV light to the substrate.
  - This will result in a dark area (i.e. non-fluorescing) area on the substrate.
  - This method is usually used in gel purification of oligonucleotides (i.e. the dark bands can be cut out with a razor blade and the oligonucleotide extracted)

Stains for Proteins

Coomassie brilliant blue

- Coomassie blue is a triphenylmethane ***textile dye*** which is able to stain proteins.
  - After a polyacrylamide gel is run it is usually "fixed" by placing in a 50% methanol/10 acetic acid solution for 30 minutes (to precipitate the proteins and prevent diffusion out of the gel).
  - The fixed gel is then soaked in a methanol/acetic acid solution containing 2.5 gm/liter of Coosmassie blue.
  - Destaining of the background gel is accomplished by soaking in changes of 10% methanol/7 % acetic acid. This method can typically detect protein samples of 0.1 ug or greater.

Silver stain

- Silver staining is a method which utilizes a silver nitrate solution to stain proteins in an acrylamide gel.
- The method is similar in nature to the use of silver in photographic plates.
- The sensitivity is approximately ***two orders of magnitude more sensitive than coomassie staining*** (i.e. one can detect approximately 1ng of protein).
- Due to its high sensitivity this method is usually used to determine the presence of trace contaminants in protein samples.

Molecular Weight Standards

- Both DNA and protein gel electrophoresis utilize molecular weight standards to calibrate the size(s) of samples being analyzed
- DNA molecular weight standards will consist of a mixture of DNA fragments of known sizes (molecular mass)
  - One convenient DNA molecular weight standard is constructed by partial ligation of a 100 base pair fragment of duplex DNA
    - Partial ligation will result in formation of dimers (200 bp), trimers (300 bp) and so on, as well as some amount of the original (100 bp) fragment
    - This produces a DNA "ladder" after gel electrophoresis
  - Another DNA fragment standard might be a known DNA sequence, such as the plasmid pBR322, which has been digested with a four-cutter restriction endonuclease (e.g. Alu I).
    - This produces a variety of fragments with different sizes
    - It is easily reproduced (just cut more pBR322)
- Protein molecular weight markers usually consist of a mixture of a half dozen or so pure proteins with known molecular masses

Protein	Molecular Mass (Da)
Phosphorylase B	94,000
Bovine Serum Albumin	67,000
Ovalbumin	43,000
Carbonic Anhydrase	30,000
Soybean Trypsin Inhibitor	20,100
a-Lactalbumin	14,400

- In both DNA and protein gels, molecular weight markers are run in a lane at one end of the gel

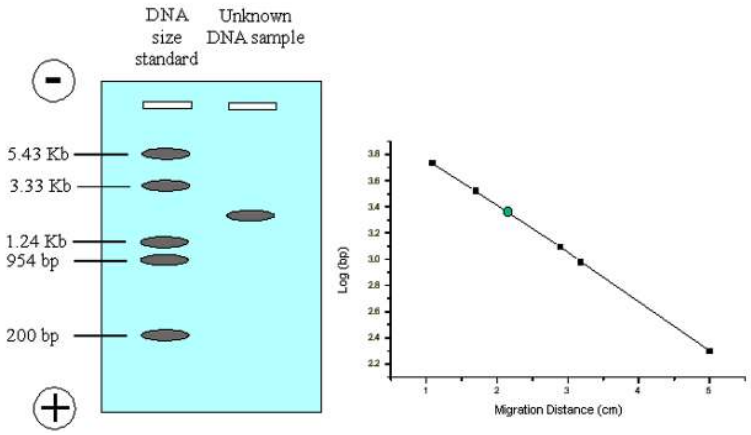


Figure 3.1.6: *DNA size standards*

This page titled 10.3.1: Gel Electrophoresis is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **3.1: Gel Electrophoresis** by Michael Blaber has no license indicated.

10.3.2: DNA Sequence Analysis

DNA sequencing is most often accomplished using a procedure referred to by one of the following names:

1. Sanger sequencing
2. Di-deoxy sequencing
3. Chain termination sequencing

- Each of these refers to the same method:
  - the use of **di-deoxy base** incorporation in a **polymerization** reaction
  - leads to termination of primer extension (a method pioneered by Fred Sanger, now retired and happily puttering about in his garden).

The basic method involves:

1. annealing a *primer* 5' to a region of DNA we would like to sequence.
2. The primer is extended in the traditional manner (i.e. with DNA polymerase and the four dNTP's).
3. However, a small concentration of *di-deoxy bases* are included in the reaction mix.
   - Usually this is accomplished by having four separate reactions, into which one of the ddNTP's is added.
   - Thus, one tube would contain primer, template, DNA polymerase, the four dNTP's and ddATP, another tube would have the same thing but with ddTTP instead of ddATP, and so on for ddCTP and ddGTP.
4. During the reaction, the normal dNTP's are incorporated into the growing chain.
   - However, occasionally the DNA pol will incorporate a *dd*base into the growing primer.
   - When this happens, the primer ***cannot*** be extended any further (because the 3' dd base does not have an available 3' hydroxyl group).
   - *The resulting DNA fragment begins at the 5' end of the sequencing primer and ends at the site of dd base incorporation*.

Thus, in the reaction mixture containing the dd base ddATP, there will result an ensemble of fragements of varying lengths, each ending in with the ddA base (i.e. at all positions in the template where there was a comlementary 'T' base).

The mixture containing ddCTP will have a different mix of fragements - they will contain ddC at the 3' ends (at positions in the template where 'G' bases were located).

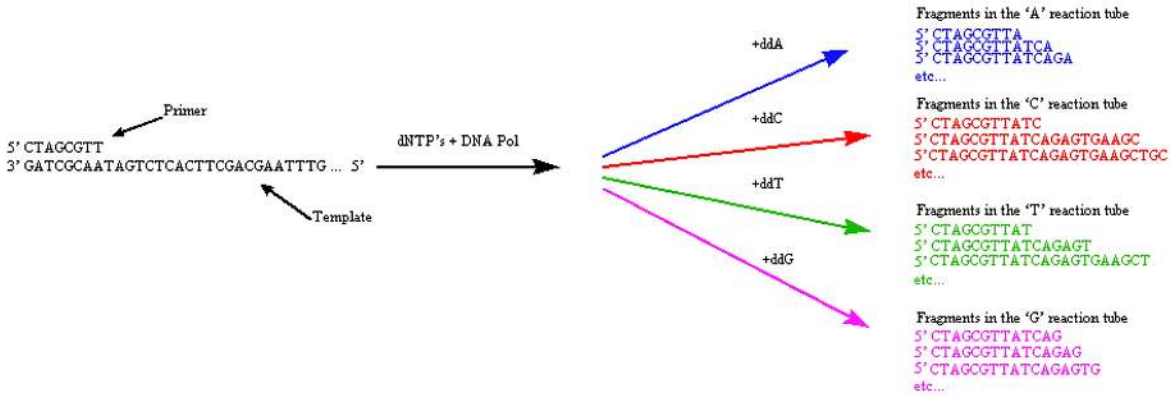


Figure 3.2.1: *DNA fragments*

- If the fragments from the 'A' reaction mix are run on a urea/acrylamide gel (typically 6%) the fragments will separate according to size.
- Likewise for the 'C', 'G' and 'T' reaction mix fragments.
  - If the four different reaction mixtures are run next to each other the fragment sizes can be ***directly compared to one another***.
  - Note that the shortest expected fragment is the primer itself and the longer the fragment, the further from the primer the extension reaction went before termination.
- Consider a case where the template has a stretch of six 'A' bases in a row.

- In the 'T' reaction mix we will subsequently get six fragments; each ending in 'T' and differing by one base length.
- None of the other reaction mixes will contain fragments between these lengths (they will either be longer or shorter) because none of the other reaction mixes will terminate within this region.
- Thus, if we run the four reaction mixes side by side and look at the fragment patterns we would see the following:

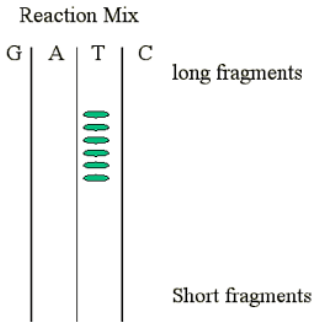


Figure 3.2.2: *Example fragment patterns with 6 A's*

Now consider a template that contains the sequence 3' GATC 5' (***note the orientation***).

- When the primer is extended in the different reaction mixes it can truncate first at the G (incorporating a dd 'C'), then at the A (incorporating a dd 'T') and so on.
- The fragments run on a gel would thus look like:

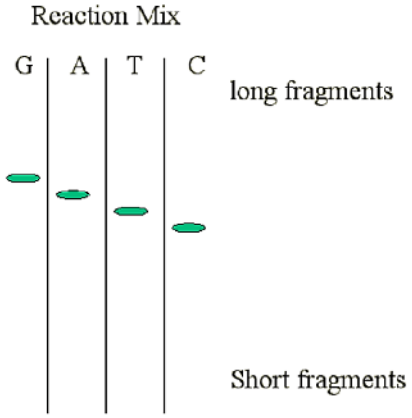


Figure 3.2.3: *Example fragment patterns with GATC*

- Thus, following the ladder of fragments on the sequencing gel allows you to "read" the sequence of the template.
- *Note however, that in regard to the template when we read from the bottom of the gel to the top we are reading in the 3' to 5' direction and reading the complementary bases to the actual sequence*.

Visualization of fragments

- If radiolabeled dATP is spiked into the mixtures it will be incorporated like a "normal" dATP base.
  - However, the resulting DNA fragment will be *radiolabled*.
  - Thus the acrylamide gel can be exposed to x-ray film and the location of the fragments determined.
- Recently, automated sequencers have made use of specific dyes which are tagged to the dideoxy bases.
  - These dyes can be "read" by a laser and thus the specific terminating dd base for a particular DNA fragment can be identified.
  - Thus, the fragments can read as they elute, rather than stopping the gel and exposing it.
  - Furthermore, since each dd base can be uniquely identified, all four reactions (ddA, ddC, ddG and ddT chain termination) can be done in a single tube and run in a single lane on a sequencing gel

    - Automated sequencers can thus read ***further*** than with manual methods.
    - Since a single lane is used per sample (as compared to four lanes with the radiolabeled method) many more samples can be analyzed and the ***throughput*** is greater
- The acrylamide gels used for sequence analysis are typically 50 cm to 100 cm long.
    - In manual sequencing the four reaction mixes are loaded and the gel is run for approximately 2 hours then the samples are reloaded on another part of the gel and the gel run is continued. A third set of samples may be loaded after another 2 hours.
        - The gel is stopped after the dye front of last sample loaded has just reached the bottom of the gel.
        - Thus, the short fragments can be visualized in the last load, medium fragements in the second load and the long fragments will be visualized in the first set of reaction mixtures loaded.
        - Manual sequencing can resolve on the order of 400 bases of continuous sequence. Automated sequencers can routinely provide twice this amount of information.
    - Automated sequencers use the same types of glass plates
        - The continuous running of the gel (and dye identification) means that typically 400-700 bases or more can be read
        - Automatic software will interpret the dye signals into a sequence
        - nuances of the sequencing chemistry and expert knowledge can be programmed into the sequence analysis software (e.g. the software can compensate for the "smile" of the gel)

This page titled 10.3.2: DNA Sequence Analysis is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **3.2: DNA Sequence Analysis** by Michael Blaber has no license indicated.

10.3.3: Polymerase Chain Reaction (PCR) and Cloning of PCR Products

PCR is an *in vitro* technique for the amplification of a region of DNA which lies between two regions of known sequence. PCR amplification is achieved by using oligonucleotide primers. These are typically short, single stranded oligonucleotides which are complementary to the outer regions of known sequence.



Figure 3.3.1: *Complementary oligonucleotide primers*

The oligonucleotides serve as ***primers*** for DNA polymerase and the denatured strands of the large DNA fragment serves as the ***template***. This results in the synthesis of new DNA strands which are complementary to the parent template strands. These new strands have **defined 5' ends** (the 5' ends of the oligonucleotide primers), whereas the 3' ends are potentially ambiguous in length.

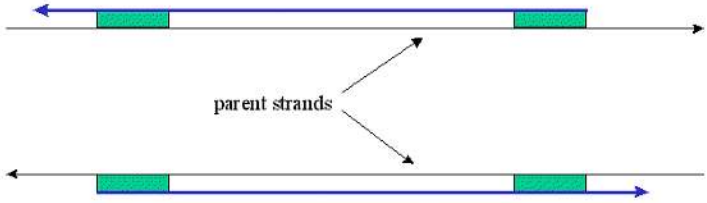


Figure 3.3.2: *Synthesis of new DNA strands*

The oligonucleotide directed synthesis of daughter DNA strands can be repeated if the new duplex is denatured (by heating) and additional primers are allowed to anneal (by cooling to an appropriate temperature).

The steps of:

1. *Template denaturation*
2. *Primer annealing*
3. *Primer extension*

comprise a single "cycle" in the PCR amplification methodology.

After each cycle the newly synthesized DNA strands can serve as templates in the next cycle.

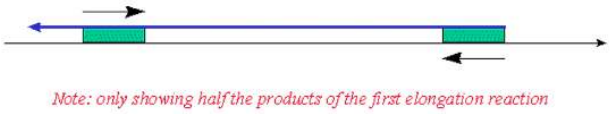


Figure 3.3.3: *Product of elongation reaction as template*

Note that half of the newly synthesized strands from the second round of replication have 5' and 3' termini which are defined by the annealing location of the primer oligonucleotides.

Summary of products at the end of each PCR cycle:

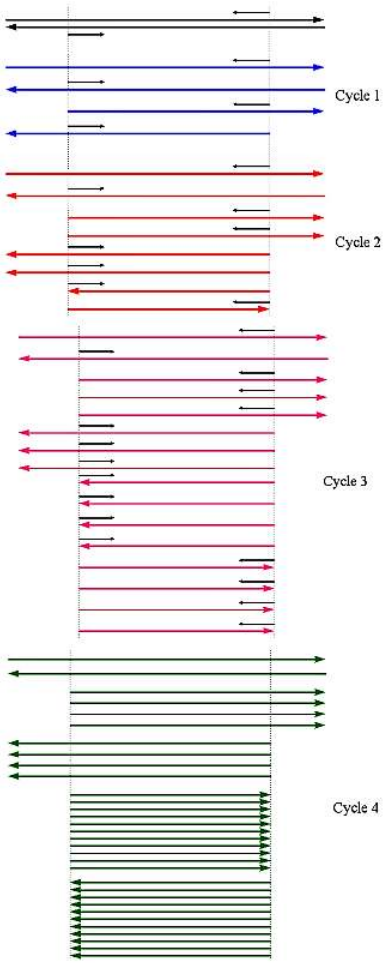


Figure 3.3.4: *PCR products*

The total of each of the different types of oligonucleotide fragments for each cycle can be summarized as follows:

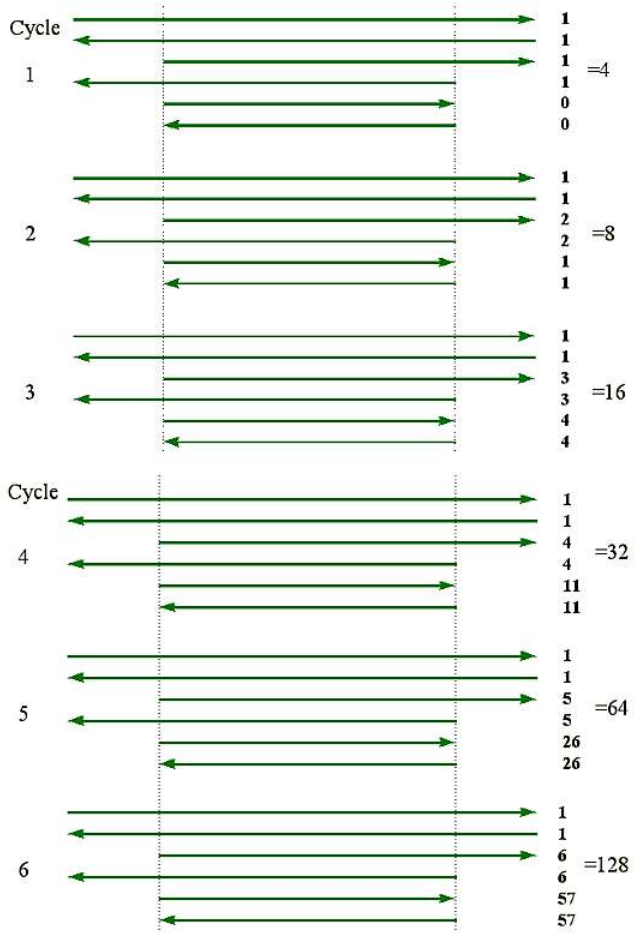


Figure 3.3.5: *Types of fragments for each cycle*

- The amplification of the fragments follows the following pattern:
  - There is always 1x copy of each of the original templates - the PCR as outlined never reproduces the full length template
  - There will be 'n'x copies of each fragment with an indeterminate length (where 'n' is the number of cycles). The fragments of indeterminant length have one end defined by a PCR primer and the other end is of indeterminant length
  - There will be $(2^n - (n + 1))$ x copies of each fragment of defined length (i.e. each end defined by the two PCR primers).
- For example, after 6 cycles we will have
  - 1 x copy of each original template
  - 6 x copies of each fragment of indeterminant length
  - (64 - (6 + 1)) = 57 x copies of each fragment of defined length
- In the above case, the desired PCR product will be a duplex of the defined length fragment. The question is: how many will be produced?
  - The original templates will not necessarily anneal with one another
  - Likewise, the templates of indeterminant length will not necessarily anneal with one another
  - Furthermore, as the number of cycles proceeds, the defined length fragments far outnumber the others
  - ***Therefore, the original template, and fragments of indeterminant length are most likely going to hybridize with defined length fragments***
  - Our expected ***amplification*** would therefore be (using the 6 cycle PCR example)

57 - (6 + 1) = 50 x copies of each defined length fragments

(i.e. 50 x duplexes of defined length)

- The expected ***amplification*** of the desired defined length product with respect to the original template concentration (x) can thus be represented by the formula:

$$[(2^n - (n+1)) - (n+1)]x \tag{10.3.3.1}$$

or

$$(2^n - 2(n+1)) \tag{10.3.3.2}$$

(this is often abbreviated to a simple rule of thumb for the ***amplification***: **(2^n - 2n) x**)

The interpretation of this formula is that

- For a given number of cycles 'n' we make '2^n x' ***total possible duplexes***
- For a given number of cycles there will be '2(n+1) (or 2n in our approximation) x' duplexes which are formed from either the original template, or a fragment of indeterminate length, along with a fragment of defined length (and represent an undesired product)
- Thus, the total concentration of desired product (duplexes with a length defined by the PCR primers) will be (2^n - 2(n+1)) x (where x is the concentration of the original duplex)
- The theoretical amplification value is never achieved in practice. Several factors prevent this from occurring, including:

1. Competition of complementary daughter strands with primers for reannealing (i.e. two daughter strands reannealing results in no amplification).
2. Loss of enzyme activity due to thermal denaturation, especially in the later cycles
3. Even without thermal denaturation, the amount of enzyme becomes limiting due to molar target excess in later cycles (i.e. after 25 - 30 cycles too many primers need extending)
4. Possible second site primer annealing and non-productive priming

> **Note**
>
> PCR was invented in 1985 by Kary Mullis, working for Cetus corporation somewhere near Berkeley, California. The original method of PCR used the Klenow fragment *of E. coli* DNA polymerase I. This enzyme, however, denatures at temperatures lower than that required to denature most template duplexes. Thus, after each cycle, fresh enzyme had to be added to the reaction. This was quite tedious. In addition to this problem with the enzyme, the samples had to be moved from one temperature bath to another to allow the individual steps of denaturation, annealing and polymerization, which all required different temperatures. This was pretty tedious too.
>
> Thus, **two main advances** allowed the process to be **automated**, these advances were:
>
> 1. The use of **thermostable DNA polymerases**, which resisted denaturation (inactivation) at high temperatures. Thus, an initial aliquot of polymerase could last throughout the numerous cycles of the protocol. The first thermostable DNA polymerase to be used was isolated from the bacterium *Thermus aquaticus*. It was isolated from a hot spring in Yellowstone National Park where it lived happily (i.e. it replicated its DNA) at temperatures in excess of 85 °C
> 2. The development of temperature baths which could shift their temperatures up and down rapidly and in an automated programmed manner. These are known as **thermal cyclers** or **PCR machines**.
>
> These two developments let to the automation of PCR. The PCR process is covered by patents owned by Hoffmann-La Roche Inc. (a faceless conglomerate you can trust).

Thermal cycling parameters

- The thermal cycling parameters are critical to a successful PCR experiment. The important steps in each cycles of PCR include:

1. denaturation of template
2. annealing of primers
3. extension of the primers

A representative temperature profile for each cycle might look like the following:

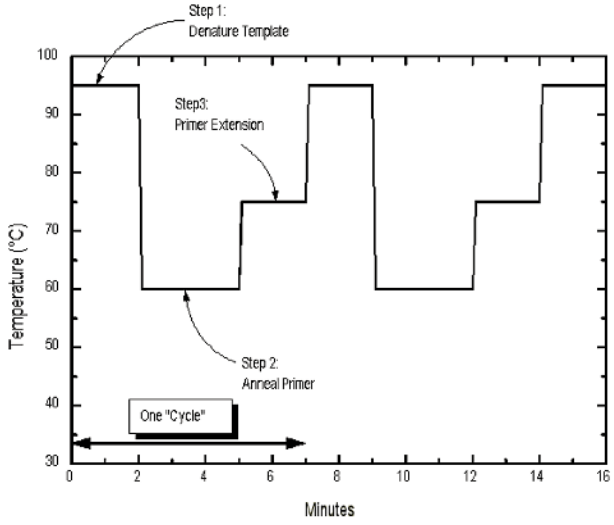


Figure 3.3.6: *Temperature for PCR cycle*

Template denaturation

- The *initial* denaturation of template is accomplished at 95-100 °C.
  - Supercoiled plasmids are tougher to melt and may require boiling for several minutes, or may be initially denatured by using base (NaOH, followed by pH neutralization).
  - Denaturation during the PCR experiment (i.e. second cycle onward) is usually accomplished at temperatures of 92-95 °C (usually empirically determined).

Primer annealing temperature

- Primer annealing temperature is an important parameter in the success of the PCR experiment.
- The annealing temperature is characteristic for each oligonucleotide:
  - it is a function of the length and base composition of the primer as well as the ionic strength of the reaction buffer.
  - Estimates of the annealing temperature can be calculated in several different ways.
  - These calculated annealing temperatures are a ***starting point*** for the PCR experiment, but ideal annealing temperatures are determined empirically.

Primer extension

- Primer extension is usually performed at 72 °C, or the *optimum temperature of the DNA polymerase*.
- The length of time of the primer extension steps can be increased if the region of DNA to be amplified is long, however, for the majority of PCR experiments an extension time of 2 minutes is sufficient to get complete extension.

Number of cycles

- The number of cycles is usually between 25 and 35.
  - More cycles mean a greater yield of product.
  - However, with increasing number of cycles the greater the probability of generating various artifacts (e.g. mispriming products).
  - It is unusual to find procedures which have more than 40 cycles.

Choice of Polymerases for PCR

- One of the important advances which allowed development of PCR was the availability of thermostable polymerases.
- This allowed initially added enzyme to survive temperature cycles approaching 100 °C.

Thermostable DNA polymerases and their sources

DNA Polymerase	Natural or recombinant	Source
Taq	Natural	*Thermus aquaticus*
Amplitaq®	Recombinant	*T. aquaticus*
Amplitaq (Stoffel fragment)®	Recombinant	*T. aquaticus*
Hot Tub™	Natural	*Thermus flavis*
Pyrostase™	Natural	*T. flavis*
Vent™	Recombinant	*Thermococcus litoralis*
Deep Vent™	Recombinant	*Pyrococcus GB-D*
Tth	Recombinant	*Thermus thermophilus*
Pfu	Natural	*Pyrococcus furiosus*
ULTma™	Recombinant	*Thermotoga maritima*

- Properties of DNA polymerases used in PCR

	Taq/Amplitaq®	Stoffel fragment	Vent™	Deep Vent™	*Pfu*	*Tth*	ULTma™
95 °C half-life	40 min	80 min	400 min	1380 min	>120 min	20 min	>50 min
5'3' exo	+					+	
3'5' exo			+	+	+		+
Extension rate (nt/sec)	75	>50	>80	?	60	>33	?
RT activity	Weak	Weak	?	?	?	Yes	?
Resulting ends	3' A	3' A	>95% blunt	>95% blunt	blunt	3' A	blunt
Strand displacement			+	+			
M.W. (kDa)	94	61	?	?	92	94	70

Buffers and $MgCl_2$ in PCR reactions

A typical reaction buffer for PCR would something like:

- 10 mM Tris, pH 8.3
- 50 mM KCl
- 1.5 mM $MgCl_2$
- 0.01% gelatin
- The $MgCl_2$ concentration in the final reaction mixture is usually between 0.5 to 5.0 mM, and the optimum concentration is determined empirically (typically between 1.0 - 1.5 mM). **Mg^{2+} ions**:
  - form a soluble complex with dNTP's which is essential for dNTP incorporation
  - stimulate polymerase activity
  - increase the Tm (melting temperature) of primer/template interaction (i.e. it serves to stabilize the duplex interaction

Generally,

- **low** Mg^{2+} leads to low yields (or no yield) and
- **high** Mg^{2+} leads to accumulation of nonspecific products (mispriming).

Primers

Primer design

- Generally, primers used are 20 - 30 mer in length. This provides for practical annealing temperatures (of the high temperature regimen where the thermostable polymerase is most active).
- Primers should avoid stretches of polybase sequences (e.g. poly dG) or repeating motifs - these can hybridize with inappropriate register on the template.
- Inverted repeat sequences should be avoided so as to prevent formation of secondary structure in the primer, which would prevent hybridization to template
- Sequences complementary to other primers used in the PCR should be avoid so as to prevent hybridization between primers (particularly important for the **3' end** of the primer)
- If possible the 3' end of the primer should be rich in G, C bases to enhance annealing of the end which will be extended
- The distance between primers should be less than 10 Kb in length. Typically, substantial reduction in yield is observed when the primers extend from each other beyond ~3 Kb.

Melting temperature (Tm) of primers

- The Tm of primer hybridization can be calculated using various formulas. The most commonly used formula is:

$$Tm = [(number of A + T residues) \times 2°C] + [(number of G + C residues) x 4°C] \quad (10.3.3.3)$$

- This formula was determined originally from oligonucleotide hybridization assays, which were performed in 1 M NaCl, and appears to be accurate in lower salt conditions only for primers less than about 20 nucleotides in length.
- The common wisdom is that the Tm is more like 3-5 °C lower than the value calculated from this formula.

$$Tms = 81.5 + 16.6(\log_{10}[J+]) + 0.41(\%G + C) - (600/l) \quad (10.3.3.4)$$

- Where [J^+] = the **molar** concentration of monovalent cations (e.g. Na^+ from NaCl), and l = the length of oligonucleotide. (%G+C) is the actual percentage value and not a fractional representation (i.e. the value to insert for a primer which had 90 % G+C content would be "90" and not "0.90").
- This formula is reportedly useful for primers of 14 to 70 bases in length.

$$Tmp = 22 + 1.46([2 \times (G + C)] + (A + T)) \quad (10.3.3.5)$$

- This formula is reportedly useful for primers of 20-35 bases in length.
- The calculated annealing temperature is only a ***reference temperature*** from which to initiate experiments.
  - The actual annealing temperature may be 3-12 °C higher than the calculated Tm.
  - The actual annealing temperature condition should be determined empirically.
  - The highest annealing temperature which gives the best PCR product should be used.
- Examples of T_m , T_ms and T_mp calculations (0.05 M K^+)

	G	A	T	C	T_m	T_ms	T_mp
15 mer	3	5	2	5	46	42	56
20 mer	6	5	4	5	62	52	67
30 mer	8	6	8	8	92	62	89

Calculating primer concentrations

- The molar concentration of a primer can be calculated based on the absorbance of the primer at 260 nm (A_{260}) and the **molar extinction coefficient** for the primer at this wavelength.
- The molar extinction coefficient for the primer can be calculated by knowing the sequence of the primer and then summing the molar extinction values for the individual bases which comprise the primer.
- The individual bases have the following molar extinction coefficients at 260 nm:
  - A 1.0 molar solution of dT has a value of 8,400 absorbance units at 260 nm.

- A 1.0 molar solution of dA has a value of 15,200 absorbance units at 260 nm.
- A 1.0 molar solution of dG has a value of 12,010 absorbance units at 260 nm.
- A 1.0 molar solution of dC has a value of 7050 absorbance units at 260 nm.

- For example, the primer 5' TAGC 3' would have a molar extinction coefficient of 42,660 at 260 nm. Likewise, a 10 micromolar solution of this primer would give an absorbence of 0.427 at 260 nm.

Cloning PCR Products

Introduction of restriction sites

- It is possible to introduce restriction site sequences into PCR products by having these sequences incorporated into the **5' end** of the PCR primer(s).

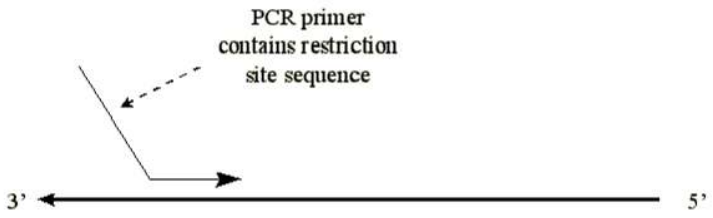


Figure 3.3.7: *Restriction site sequence introduction*

- The short restriction site sequence on the 5' end of the PCR primer will not hybridize, but as long as the 3' hybridizing region is long enough (i.e. its Tm is high enough; ~20 mer) the primer will specifically bind to the appropriate site.
- The PCR product will thus have an additional DNA squence at the 5' end which will contain the endonuclease restriction site.
- A similar or different restriction site sequence can be added via the other PCR primer.
- If the other primer has a different restriction sequence then the PCR fragment can be inserted in a directional dependent manner in a host plasmid.

The potential problems with this method include:

- There is no easy way to prevent internal sites containing similar restriction sequences from being cut when the end of the PCR product are cut
- Restriction sequences are inverse repeat sequences, thus the potential exists for primer dimer association and resultant non-productive annealing

Generation of half sites

- This method is similar to the method of introducing restriction sites, described above.
- The primary difference is that instead of the primer containing the entire restriction site sequence (say the six nucleotides of a six cutter) it will contain only the last three (and the other PCR primer will contain the complementary sequence for the first three).

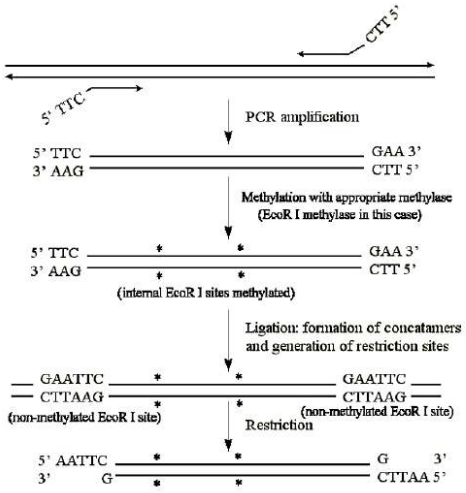


Figure 3.3.8: *Half sites*

The advantages of this method are:

- Typically internal restriction sites cleave with much greater efficiency (i.e. some sites if located at the ends of linear DNA never cut well at all)
- There is no need to gel purify linker fragments after digestion
- The DNA can be methylated (the half sites will not be). After concatenation the linkers will be cut but internal restriction sites will not
- A disadvantage is that the same restriction site is incorporated into both ends so the PCR fragment cannot be ligated into a host vector in an orientation dependent manner.
- Also, in this method 3' A overhang cannot be tolerated.

Blunt end ligation

- As stated earlier some thermostable DNA polymerases add a single dA residue onto the 3' end of the PCR product.
- There are three choices to be made when attempting to subclone without the use of added restriction sites within the primers:
  - Use a DNA polymerase which leaves the 3' strand blunt (e.g. Vent) and do a blunt end ligation (i.e. host vector was opened up with blunt cutting restriction endonuclease)
  - "Fix" the 3' A overhang by chewing back with Pol I, dNTP's.
  - Use the 3' A overhang to anneal and ligate to a "T" vector - a vector which has a single dT overhang on its 3' ends.
- "T" vectors can be made by opening up a vector, via a blunt cutting restriction endonuclease and ligating in a specific linker.
- The linker contains a restriction sequence for a restriction endonuclease which recognizes an interrupted palindrome and cuts in the internal region with a single 3' overhang.
- The linker will contain two copies of the restriction recognition sequence, the first with a sequence in the interrupted palindrome which leaves a 3' T overhang and the second with a sequence in the interrupted palindrome which leaves another 3' T overhang
- This may sound confusing, so here's an example of how to make a T vector:

Ahd I restriction endonuclease:

```
          |
G A C N N N N N G T C
C T G N N N N N C A G
          |
```

Ahd I will cut this sequence to produce:

```
G A C N N N                    N N G T CC T G N N
```

We could design an oligonucleotide with two Ahd I restriction sequences, with slightly different sequences in the interrupted region of the palindrome, to give:

```
            |                        |
G A C N N T N N G T C G A C N N A N N G T C
C T G N N A N N C A G C T G N N T N N C A G
            |                        |
```

If this this were inserted into a vector, and the vector then was cut with Ahd I, it would have the following sequence at the ends of the linearized vector:

```
-G A C N N T                      N N G T C-
-C T G N N                      T N N C A G-
```

In other words, a 3' 'T' overhang at both ends of the vector. A PCR product, with 3' A overhangs could thus be inserted into such a 'T' vector

Adding promoters, ribosome binding sites, start codons, and stop codons

- The ability to add unique sequences to the 5' ends of PCR primers allows for short control elements to be ***directly incorporated***.
- These can include a start codon or stop codon (3 bases), a promoter (~30 nucleotide region) or a ribosome binding site (~8 bases).

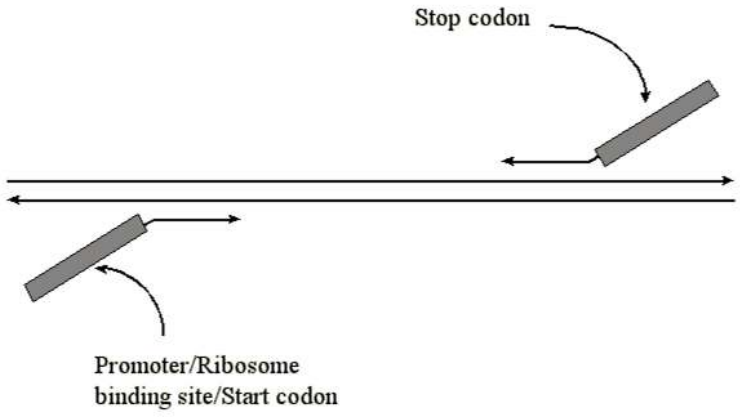


Figure 3.3.9: *Inserting promoter/ribosome/binding site/start codon/stop codon*

PCR Mutagenesis

Gene fusion

- This method is useful for joining overlapping regions of a large gene, or for the construction of **chimeric** genes.

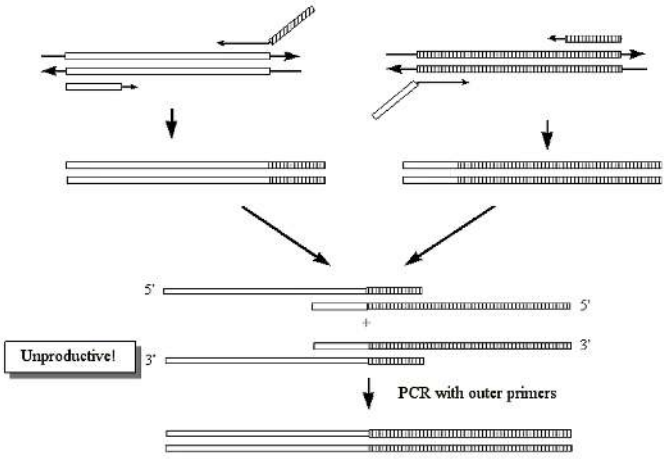


Figure 3.3.10: *Gene Fusion*

Creation of deletions within a gene

- A very similar methodology can be used within a single gene for the production of a mutant gene containing a specific deletion:

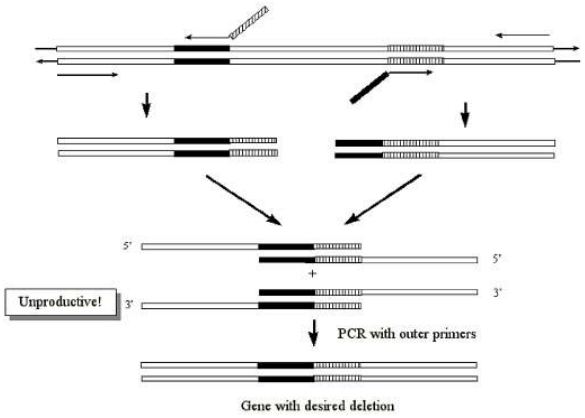


Figure 3.3.11: *Deletions*

- If the gene is contained within circular DNA (i.e. a plasmid) deletions can be constructed in a single PCR reaction with a single set of primers (this type of methodology is also known as **"inverse" PCR**).

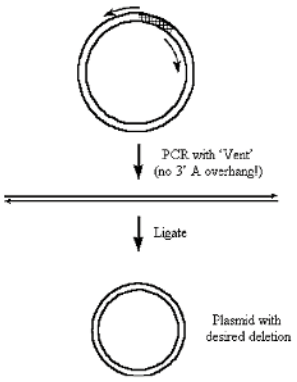


Figure 3.3.12: *Inverse PCR*

Generation of point mutation(s) - i.e. base substitution mutations

- The generation of base substitutions can proceed along a similar route as with the deletion mutations.
- However, in this case the primers are mutagenic - there will be a mismatch, or mismatches, between the primer and target sequences.
- The mutagenic oligo will have a lower than expected Tm due to this mismatch(es).

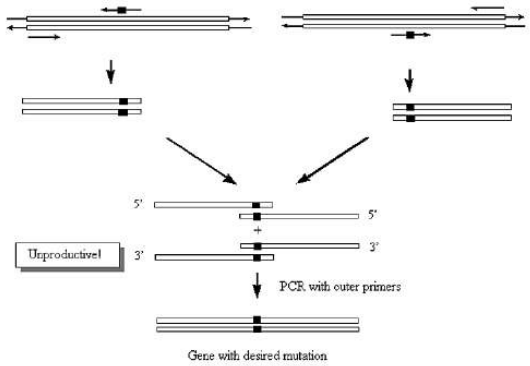


Figure 3.3.13: *Point mutation*

Introduction of base substitutions via asymmetric PCR:

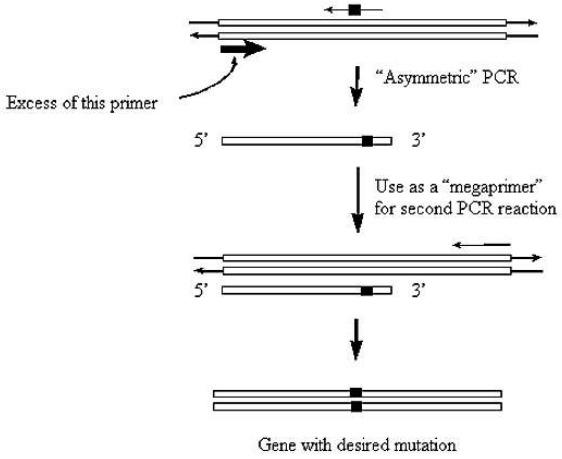


Figure 3.3.14: *Substitution with asymmetric PCR*

Insertion mutagenesis

- Short insertions (~1-6 basepairs) can be incorporated directly into a PCR primer, either internally, or at the 5' end.
- If the template DNA is linear and the desired site of insertion is not at the end of the template, then the entire gene (plus insertion) can be produced using asymmetric PCR or overlapping PCR (i.e. shown above).

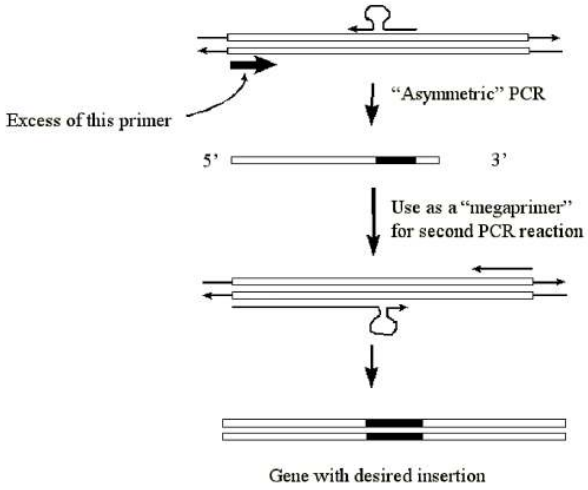


Figure 3.3.15: *Insertion mutagenesis*

- Large insertions can be accomplished by using a template (the desired insertion) for PCR with the primers having 5' sequences which are complementary to the region of insertion in the desired gene:

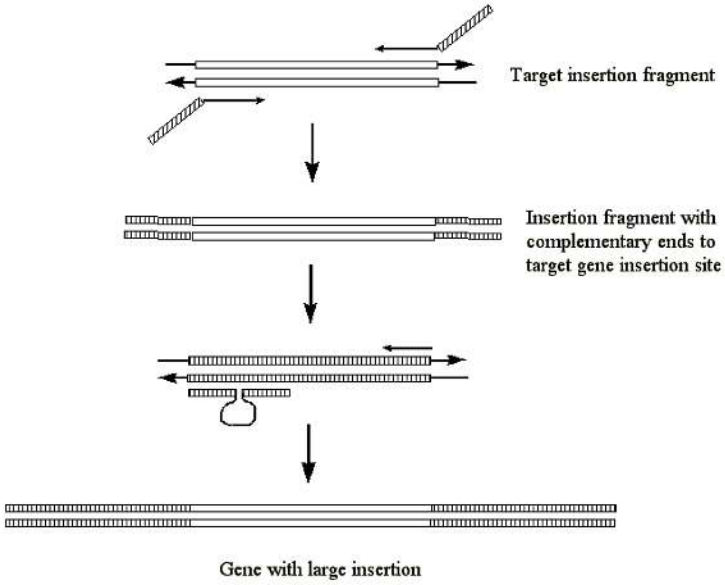


Figure 3.3.16: *Large insertion*

"Random" mutagenesis with PCR

- The PCR protocol can be modified so as to introduce mutations at random positions in the target DNA.
- The principle behind the mutagenesis is **misincorporation** of bases at "random" positions.
- Misincorporation by Taq polymerase, for example, can be achieved by adding Mn^{2+} to the reaction buffer, and decreasing the concentration of one of the four dNTP's.
  - At the sites in the template where the reduced base should be incorporated, there will be an increased probability of misincorporation.
  - Thus, the choice of base with diminished concentration determines the sites in the template which will potentially be mutated.
  - The misincorporated base is more or less random.

- The ideal Mn^{2+} concentration to add varies between 0.1 to 0.5 mM and is determined empirically. The relative concentrations of bases is 1 mM for each base, except the reduced base, which is typically present at a 1:5 or 1:10 ratio (i.e. 0.2 to 0.1 mM).

This page titled 10.3.3: Polymerase Chain Reaction (PCR) and Cloning of PCR Products is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **3.3: Polymerase Chain Reaction (PCR) and Cloning of PCR Products** by Michael Blaber has no license indicated.

10.3.4: Prokaryotic Expression Vectors

The first vector we will consider is the *pUC* family of vectors

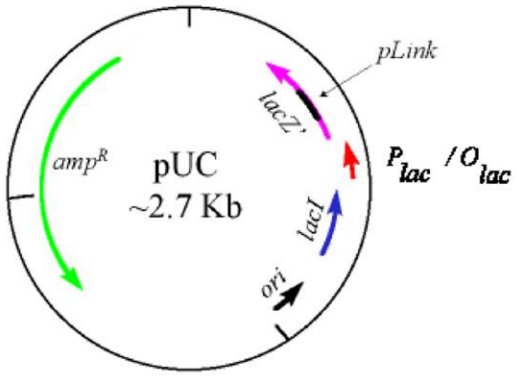


Figure 3.4.1: *pUC vector*

Although it is not typically used for the expression of recombinant proteins, it has all the necessary elements of an expression vector:

1. *An origin of replication*. The pUC family of vectors are *high copy* vectors. They have a ColE1 origin of replication, but a deletion of the *rop* replication regulatory region.
2. *A drug resistance marker*. The pUC vectors contain a gene for *ampicillin resistance* (b-lactamase).
3. *An inducible promoter*. This family of vectors contains the *lac* promoter (P_{lac}) along with the associated *lac* Operator region (O_{lac})

These vectors contain a couple of other elements which add utility to the vector:

1. *The lacI gene*. This gene codes for the *lac* repressor protein. Being a high copy plasmid the host *lac*repressor levels may not be sufficient to efficiently repress the *lac* operator on the plasmids. The plasmid produces *lac* repressor to augment the host levels.
2. *The lacZ' gene*. This gene product is transcribed from the *lac* promoter and produces an amino-terminal fragment of the b-galactosidase protein.
3. *A polylinker (multiple cloning site)*. This short stretch of DNA is located just downstream from the *lac*promoter and after the first few codons of the *lacZ'* gene. It is a short stretch of nucleotides which contains a variety of restriction endonuclease sites.

a-complementation

The *lac* promoter of the pUC plasmid can be induced using the lactose analogue *isopropylthiol-b-D-galactoside*. ("IPTG").

- This will result in the expression of the lacZ' gene.
- This gene codes for an amino-terminal fragment of the b-galactosidase protein
- By itself, this fragment is *non-functional* (it will not hydrolyze lactose or other b-galactosides)

However, b-galactosidase is an interesting protein:

- If we express the carboxy-terminal fragement of the protein it is also non-functional
- But, if we combine both peptide fragments they can combine to yield a *functional* b-galactosidase protein
- This is termed ***a-complementation***

The *lac*ZDM15 mutation in a bacteria means that its ***genome*** has suffered a deletion of the amino-terminal region of the lacZ gene.

- Such a bacteria has a *non-functional* b-galactosidase protein
- If this type of bacteria contains an extra-chromosomal element which expresses the *lacZ'* peptide, it can complement the *lac*ZDM15 protein to produce functional b-galactosidase protein (*a-complementation*)

The galactoside, *5-bromo-4-chloro-3-indoyl-b-D-galactoside* ("X-gal"), can be hydrolyzed by b-galactosidase and produces a **dark blue** color.

- *lacZ*DM15 bacteria in the presence of lactose (or IPTG) and X-gal will appear a normal white-yellow color.
- The same bacteria which harbor the above pUC plasmid will be dark blue in color when IPTG and X-gal are present in the media (due to a-complementation and a functional b-galactosidase protein)

The pUC polylinker region

The pUC polylinker region is a short stretch of DNA which is actually inserted *within* the *lacZ'* gene, just downstream from the start codon:

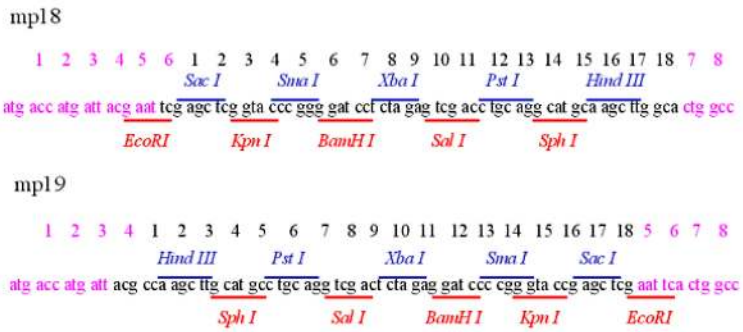


Figure 3.4.2: *pUC Polylinker region*

> **Note**
>
> In the above diagram the codon numbers for the wild-type b-galactosidase are given in magenta (in this case the initiator methionine is not present on purified protein and the first codon is considered to be the ACC (threonine) residue). The codons from the polyliner (which interrupt the b-galactosidase gene) are given in black.

- The different available plink regions are all a multiple of 3 nucleotides in length, therefore the lacZ' gene remains *in-frame*.
- The resulting *lacZ'*/plink peptide *can still function in a-complementation.*

What would happen to the lacZ' peptide if we inserted a DNA fragment into the plink region?

- The insertion of a random piece of DNA into the plink region will result in *three possible different reading frames* after the insert (depending on the number of nucleotides in the inserted fragment)
- There is a 33% (one in three) possibility that the downstream *lacZ'* gene will be translated in the correct reading frame
- There is also the possibility that even if the reading frame is correct that the insertion of a large peptide at the start of the *lacZ'* protein will prevent a-complementation.
- Therefore, *most of the time*, a DNA fragment inserted into the plink region will *abolish b-galactosidase function*
- DNA inserts in the plink region can therefore be identified by growing the host bacteria on media containing IPTG and X-gal and looking for white-yellow colonies (with the caveat that some blue colonies may in fact contain an insert)

DNA coding for a gene of interest can be inserted in the plink region in-frame with the lacZ' reading frame.

- The protein coded for by this gene can be expressed by induction with IPTG (i.e. using the P_{lac})
- The expressed protein will have as its amino-terminal sequence the first few amino acids of the b-galactosidase gene (in the absence of any other manipulations/mutagenesis). This is also known as a (short) *fusion protein*.
- Although the *lac* promoter is considered to be relatively weak, the high copy number plasmid may result in a useful level of expression of the protein of interest

The pET vector system (Novagen, Inc.)

The pET vector looks like this:

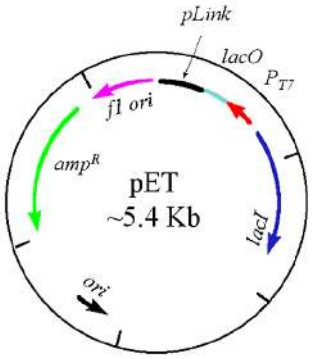


Figure 3.4.3: *pET Vector*

It has the following important elements:

- Ampicillin resistance marker
- ColE1 origin of replication
- f1 origin of replication (allows single stranded vector to be produced when co-infected with M13 helper phage)
- *lacI* gene (*lac* repressor protein)
- T7 transcription promoter (specfic for phage T7 RNA polymerase)
- *lac* operator region 3' to the T7 promoter
- multiple cloning site (polylinker region) downstream of the T7 promoter

The pET vector is a little different from the pUC vector: pUC uses the *lac* promoter and pET uses a promoter from phage T7

- The phage T7 promoter is stronger than the *lac* promoter
- Phage T7 RNA polymerase will specifically recognize the T7 promoter region and will not efficiently transcribe from other promoters
- The T7 promoter will not be efficiently transcribed by *E. coli* RNA polymerase

Where will the phage T7 RNA polymerase come from?

The pET system involves not only an expression vector, but also a *genetically engineered host bacteria*. The host bacteria for the pET vector is typically *E. coli* strain *BL(DE3)*

- This strain has integrated into its chromosome the gene for T7 RNA polymerase
- The T7 RNA polymerase in the host genome is constructed such that it is under the control of a *lac* promoter and operator
- Thus, induction by the lactose analogue, IPTG, causes the host to produce T7 RNA polymerase
- The *E. coli* host genome also carries the *lacI* (repressor) gene

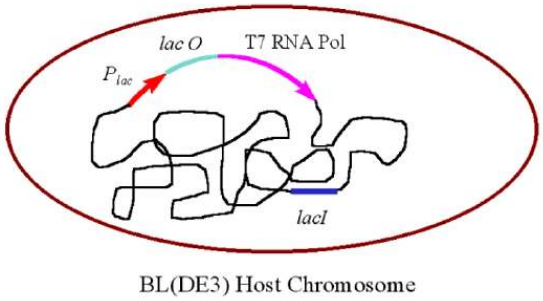


Figure 3.4.4: *E.Coli BL(DE3) chromosome*

Thus, induction by IPTG results in:

- Derepression of T7 RNA Pol gene on host chromosome with subsequent production of this polymerase
- Derepression of target gene under *lac O* regulation

- Transcription of target gene by T7 RNA Pol

Thus, the system couples a strong promoter with tight regulation (i.e. extremely low level of expression in the repressed state)

The pET vector itself is available with several different polylinker sequences. They contain the same restriction sites, but differ in the *reading frame* leading into the pLink region:

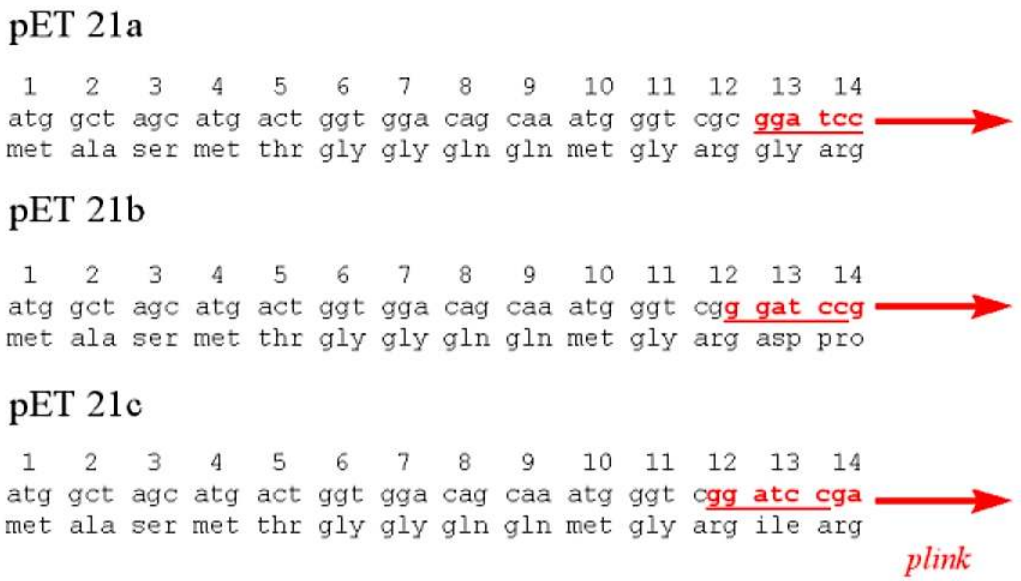


Figure 3.4.5: *Polylinker sequences*

- The *ggatcc* site (BamH I restriction endonuclease site) is the first restriction site in the polylinker.
- The polylinker region is thus available in the three different possible reading frames
- A gene of interest can thus be cloned to be *in-frame* with the transcribed region downstream from the T7 promoter. Again, like the pUC vector the expressed protein will be a fusion protein (with 12-13 amino acids at the amino-terminal end of the polypeptide)

The pTrcHis vector system (Invitrogen, Inc.)

The pTrcHis vector looks like this:

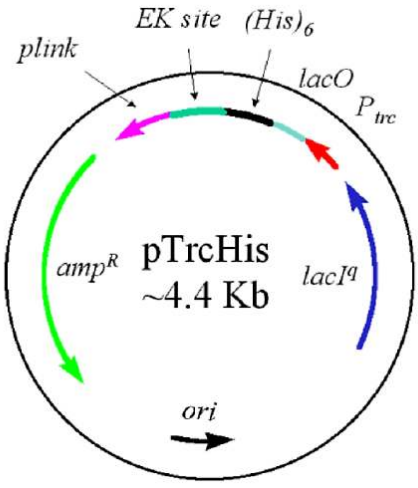


Figure 3.4.6: *pTrcHis Vector*

The important elements are:

- Ampicillin resistance marker
- ColE1 origin of replication
- *lacI*^{q} gene. This is a *lac* repressor mutation which is *upregulated* (produces more *lac* repressor than normal)
- *Trc* promoter. This is a hybrid of the *lac* and *trp* promoters which is a stronger promoter in comparison to the *lac* promoter
- The *lac* operator region downstream of P_{trc}. This allows regulation by IPTG.
- An initial stretch of 6 histidine residues in the amino terminal region of the translated protein, also known as a "*His tag*" region.
- An enterokinase (*EK*) cleavage recognition sequence (*asp asp asp asp lys* , with cleavage after the *lys* residue)
- A polylinker region downstream from the EK site

Purpose of the His tag

- Histidine residues can coordinate to form a metal (Ni^{2+}) binding site in a protein.
- The stretch of six His residues forms such a binding site.
- Proteins with a His tag have affinity for *metal chelating resins*, and this characteristic can be used to *selectively purify* such proteins.

Purpose of the EK cleavage site

- Although the His tag can allow rapid and selective purifiction of a cloned protein, the presence of these his residues may prevent normal function of the protein
- The His tag can be cleaved away from the protein by introducing a specific recognition sequence for an endopeptidase
- The sequence "asp asp asp asp lys" is recognized and cleaved by enterokinase. This sequence is not common and it is doubtful that the protein of interest contains another such sequence



Figure 3.4.7: *Enterokinase cleavage*

- His tags/EK sites are typically introduced at the amino terminus of proteins, but can be introduced at the carboxyl terminus as well

Expression hosts

One of the important characteristics of expression hosts (bacteria) is that they allow the expressed protein to accumulate.

- Some expressed proteins may not be folded correctly. Although they may be refolded later to yield functional protein, misfolded proteins may be rapidly proteolyzed in the host bacterium
- Host proteases may selectively cleave precursor forms of expressed proteins to produce mature active forms, which may not be desirable.
- *OmpT* and *lon* genes code for proteases which can degrade expressed proteins.
- Two common mutations in *E. coli* to eliminate the action of host proteases includes the *OmpT*$^{-}$ and *lon*$^{-}$ strains.
- *E. coli* strain BL(DE3) is both *OmpT*$^{-}$ and *lon*$^{-}$

This page titled 10.3.4: Prokaryotic Expression Vectors is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **3.4: Prokaryotic Expression Vectors** by Michael Blaber has no license indicated.

10.3.5: Protein Sequencing, Peptide Mapping, Synthetic Genes

Historically, some important disease states were identified as being caused by the lack of an important **protein**, or the presence of a dysfunctional mutated form of a protein.

- For example, **diabetes**, types of **dwarfism** and **hemophilia** were found to be due to deficiencies in **insulin**, **growth hormone**and **clotting factor VIII**, respectively.

These diseases could be treated by injecting **supplemental** doses of purified, or partially purified, preparations of these proteins.

- These proteins were isolated from natural materials, e.g. pig (insulin), human cadaver pituitaries (human growth hormone) or blood fractions pooled from normal donors (factor VIII).
- In most cases, even if the protein was found in relatively abundant supply, **the cost of production was substantial**.

More often than not, interesting bioactive properties were associated with proteins which could be isolated only in **minute quantitites**(e.g. the blood clot dissolving protein **tissue plasminogen activator**).

Also, **non-human proteins** typically elicited **an immune response** when injected into humans, thus the **human form** of a protein was the only useful form.

- If the protein were not readily available from blood, or urine, it would prove impractical to obtain **adequate starting material**for production.
- Unfortunately, if the material were derived from human sources, the possibility existed for the spread of **human disease** (e.g. hepatitis and the AIDS virus).

If the genetic information for these proteins could be isolated, and then transcribed and translated in an easily scaleable biological system, potentially large amounts of protein could be obtained - and hopefully, relatively cheaply.

With the development of "molecular biology", i.e.

- the structure of DNA,
- the elucidation of the genetic code,
- the identification of transcriptional promoters and ribosome binding sites,
- the isolation of restriction endonuclases,
- the identification of the origin of DNA replication
- the development of plasmids with selectable markers, and
- the culturing of *E. coli*,

the possibility existed in the mid 1970's to put it all together and produce relatively large amounts of **any human protein** for therapeutic use.

How would you go about the process of producing large amounts of some important human protein? (i.e. ***protein purification****)*

The starting point is typically **an assay for a functionality of interest**. For example, we may have a hemophiliac whose blood does not clot. However, we find that if we take a sample of his blood and add to it a small amount of blood from a "normal" individual, the hemophiliac's blood will now clot. This will be the basis forour **assay**.

Using this assay, we will fractionate normal blood using various means - chemical precipitation (with ethanol, or ammonium sulfate), and then various liquid chromatography steps, etc.

- Along the way we will follow where our clotting activity is going.
- Hopefully, at some point we will be unable to fractionate it further and will have a **pure protein**.

Once we have a pure protein we can begin to characterize it with regard to its **amino acid sequence**. From there we can ultimately get the gene for the protein and express it.

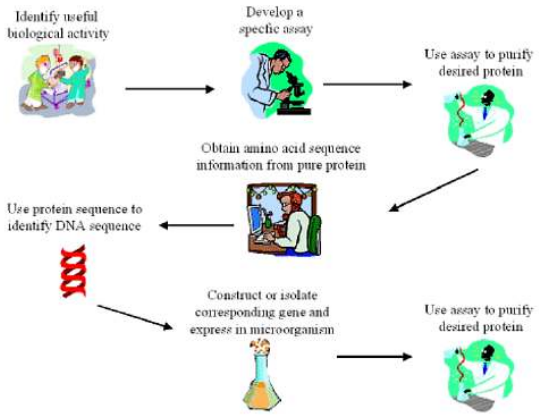


Figure 3.5.1: *Protein production*

N-terminal peptide sequence analysis

Polypeptides can be sequenced from their amino-terminus by automated procedures based upon the **Edman degradation** reaction:

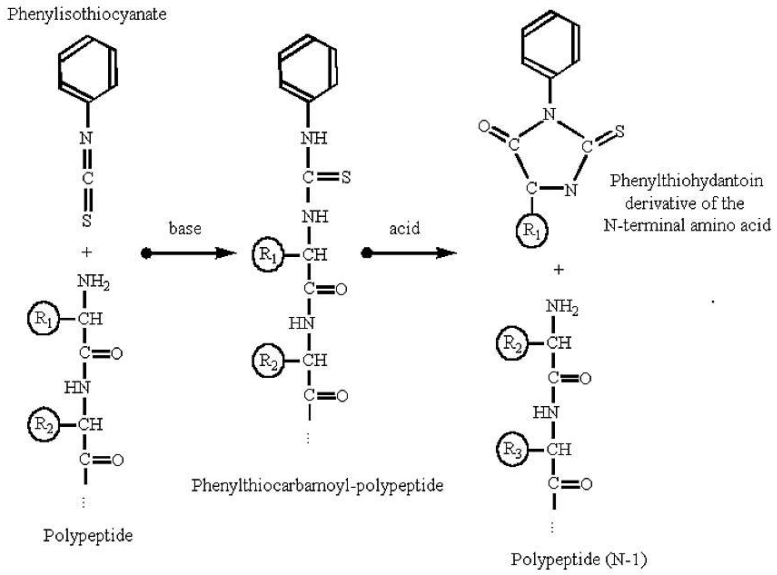


Figure 3.5.2: *Edman degradation*

- Note that with Edman chemistry only the N-terminal residue is attacted and removed, the rest of the polypeptide remains intact after the reaction.
- The new amino terminal group (previously the second amino acid in the polypeptide chain) is now available for another round of reactions. Thus, the method can be **automated**.
- The amino acid side chain of the phenylthiohydantoin derivative can be identified using liquid chromatography. Modern amino acid sequencers can probably sequence on the order of two to three dozen cycles (amino acids) of a polypeptide**.**
- *Note that the reaction requires a free amino group on the N-terminal of the protein*. If the amino-terminal residue is methylated or formylated then the reaction will not proceed (and the polypeptide is said to have a "blocked" N-terminal).

C-terminal peptide sequence analysis

C-terminal peptide sequence analysis is not as well developed as amino terminal analysis.

- The method usually makes use of **non-specific carboxypeptidases**.

- Carboxypeptidases will **sequentially** hydrolyze polypeptides from the **carboxy-terminus** end. The released amino acid can be identified using liquid chromatographic methods, and the remaining polypeptide is available for further reactions.
- Various carboxypeptidases are available, usually they are not entirely non-specific (i.e. they have certain preferences):

Name	Source	Specificity
Carboxypeptidase A	Bovine Pancreas	Aromatics, aliphatics (hydrophobics)
Carboxypeptidase B	Pig Pancreas	Arginine, Lysine, Ornithine
Carboxypeptidase P	Penicillium	Generally non-specific
Carboxypeptidase Y	Yeast	Aromatic, aliphatics

Sometimes the choice of which carboxypeptidase to use is based upon the expected sequence information. In these types of experiments:

1. samples are taken at different time points during the digestion
2. free amino acids are separated from polypeptides
3. the released amino acids are identified via amino acid analysis (liquid chromatography).

C-terminal analysis is usually only accurately for identification of the last half-dozen residues or so in a polypeptide.

Peptide Mapping

One of the obvious problems with protein sequencing is that even if the N-terminal is not "blocked" only limited sequence infomation can be obtained from an intact polypeptide (i.e. only about two dozen from the N-terminal and half a dozen from the C-terminal).

How can sequence information for the entire polypeptide be obtained?

One method is that of **peptide mapping**. Peptide mapping makes use of **proteolytic cleavages** of the polypeptide to produce smaller polypeptides. These smaller polypeptides can then be isoloted from one another and subject to sequence analysis.

How do we order the different sequences which we obtain?

One of the easiest ways is to repeat the experiment, but with a protease with a different specificity, and in this way obtain **overlapping sequence information**.

Name	Source	Specificity
Chymotrypsin	Bovine Pancreas	Cleavage after Tyr, Phe and Trp; some cleavage after Leu, Met and Ala
Bromelain	Pineapple	Cleavage after Lys, Ala and Tyr
Trypsin	Bovine Pancreas	Cleavage after Arg, less after Lys
V8 protease	*Staphylococcus aureus*	Cleavage after Glu, less after Asp

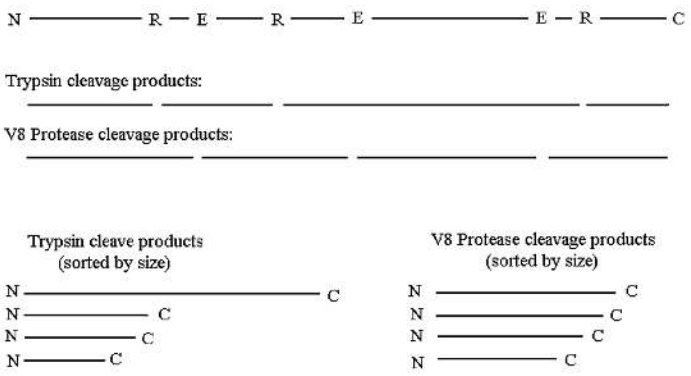


Figure 3.5.3: *Overlapping cleavage products*

Overlapping sequence information can allow you to align the peptides in the correct order and determine the sequence of the original large polypeptide (i.e. protein).

One problem which can arise deals with **Cysteine** residues and the nature of any **covalent disulfide bridges** in the protein.

- Any "peptide" mobilities (on either liquid chromatographic or PAGE analyses) which split into two smaller peptides after treatment with a reducing agent (such as b-ME) *indicate the presence of a cysteine mediated disulfide bond*.
- Upon sequencing these peptides should each contain a cysteine residue. If each peptide has only one cysteine then the disulfide bond assignment is unambiguous.

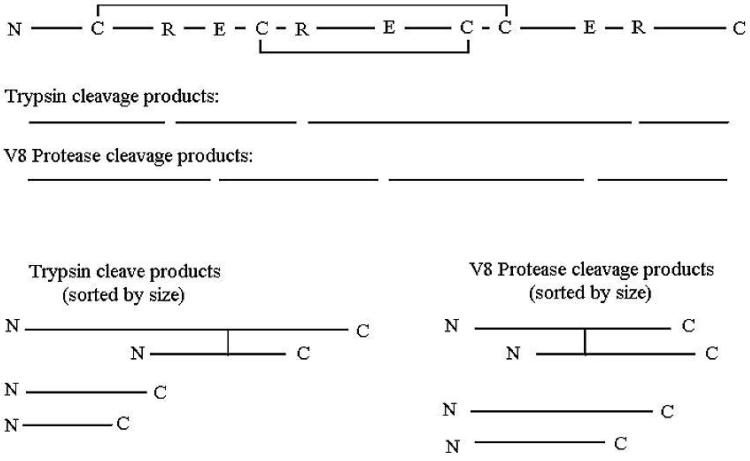


Figure 3.5.4: *Cysteine residues in cleavage products*

Corresponding genetic information

Once we have partial, or complete, peptide sequence information we can begin to identify and isolate the corresponding **genetic information**. *This is the main goal.* Once we have the corresponding genetic information it may be possible to produce relatively large amounts of the desired polypeptide.

Back translation

Since we know the genetic code, we can back translate any polypeptide sequence into a corresponding genetic sequence.

- Thus, from the amino acid sequence we could synthesize an **artificial gene** which would code for the protein of interest.

- Since many amino acids are coded for by more than one codon, there is potential **ambiguity** with regard to the original exact genetic sequence.

Amino Acid	Number of Codons
Met, Trp	1
Phe, Tyr, His, Gln, Asn, Lys, Asp, Glu, Cys	2
Ile	3
Val, Pro, Thr, Ala, Gly	4
Leu, Arg, Ser	6

However, making sure we back translate in such a way as to faithfully duplicate the original genetic sequence may not be critical - a correct protein sequence is the overall goal.

In fact, if we are attempting to express the protein in another organism (say expressing a mammalian gene in a bacterial system) we may actually prefer to choose a **codon bias** appropriate for the **expression host organism**.

Synthetic genes for small proteins are a reasonable way to proceed; this is one way in which **human insulin** has been expressed in bacterial systems.

- However, automated synthesis of DNA oligonucleotides is practical for polymer lengths of approximately 60-90 bases or less (about 20-30 amino acids).
- Furthermore, the method of construction of synthetic genes typically calls for overlapping complementary oligonucleotides (to be ligated into a single duplex DNA gene "cassette").

Thus, many oligonucleotides are required for even a single small synthetic gene.

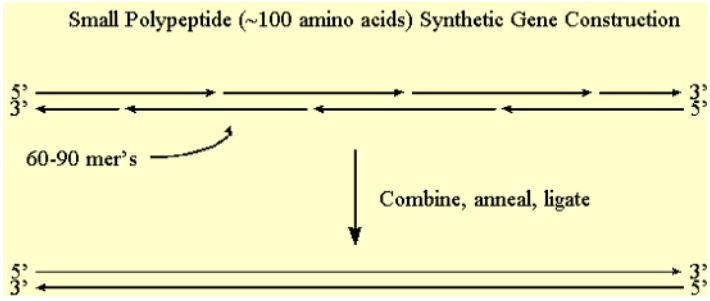


Figure 3.5.5: *Synthetic gene construction*

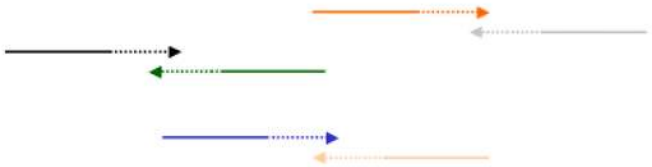
One way to improve upon the above method of synthetic gene construction is with a *direct PCR* approach. This method does not utilize ligase, or even oligonucleotides that butt together. Instead, with this method many (~100) different overlapping oligonucleotides are simultaneously used in a PCR reaction. Their sequence complementarity can be represented as follows:



The entire set of oligonucleotides may not line up to give the entire gene, but that is alright. We will do multiple rounds of PCR with the idea that *some* complementary oligo's will anneal and be extended and will lead, bit by bit, to construction of a contiguous synthetic gene:



On the next PCR cycle, some of these extended fragments will anneal with others:



These will be extended via the PCR and can go on to anneal with other larger PCR fragments. Eventually, the entire gene will be constructed. However, since the efficiency of construction of the full-length gene is probably not going to be very good, we need to conduct a subsequent PCR experiment to amplify the full-length gene (using outer primers). The principle features of this method are summarized as follows:

- Many (as many as 1-2 hundred) overlapping oligo's are combined in a single PCR reaction
- The oligos are designed to be as long as possible (~100mers) with limited overlap (~20 bases)
- The full-length gene is constructed in an initial (low yield) PCR experiment
- This full length gene is amplified with a subsequent typical PCR experiment using outer primers.

This page titled 10.3.5: Protein Sequencing, Peptide Mapping, Synthetic Genes is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **3.5: Protein Sequencing, Peptide Mapping, Synthetic Genes** by Michael Blaber has no license indicated.

10.3.6: cDNA and Genomic Libraries

Isolation of corresponding genetic information

Instead of synthesizing a desired gene, can we used the amino acid information to directly isolate the corresponding genetic information?

- There are two general sources of genetic information:

1. Genomic DNA
2. mRNA

If we are considering **genomic DNA** from eukaryotes, then there are a couple of things to consider:

1. The coding region for a gene of interest may be *interrupted* by one or more *intron* regions, and thus the complete coding region could be quite long.
2. To a first approximation, it does not matter which tissue we use to isolate the genomic information, i.e. the genomic content is the same in all tissues.

If we are considering mRNA from eukaryotes, we may realize the following advantages:

1. Introns will be spliced out and *the mRNA will contain a contiguous coding region*.
2. Tissue specific expression of the protein of interest may allow us to isolate appropriate mRNA at enhanced levels, i.e. *in tissues where the protein is expressed the mRNA levels are considerably higher than the corresponding genomic levels (there are many more molecules of mRNA than copies of the gene).*

Libraries

A "library" is a convenient storage mechanism of genetic information.

- They are typically either "genomic" or "cDNA" (i.e. mRNA in DNA form) genetic information.
- Deduced genetic sequences from corresponding polypeptide information can be used to identify specific genetic information within a library.

cDNA library construction

"Reverse transcription" is a mechanism whereby genetic information contained in mRNA is converted back into a double stranded DNA form.

The enzyme responsible for this is an RNA dependent DNA polymerase called **reverse transcriptase**.

- Reverse transcriptases have traditionally been isolated from viruses whose genome is actually in an RNA form and must be converted to duplex DNA.
- These viruses typically carry a functional reverse transcriptase along with their mRNA genetic component when they infect cells.
- One of the most common commercially available reverse transcriptases **is Moloney murine leukemia virus** (MMLV).
- This *RNA dependent DNA polymerase* (as will all polymerases) add nucleotides to a nacent polynucleotide in the 5' to 3' direction using RNA as the *template*. It does not contain any 3'->5' exonuclease (proofreading) activity.

MMLV will use mRNA as a template, but requires a **primer** (it can extend a DNA primer but cannot synthesize one).

- One of the really neat things about eukaryotic mRNA's is the presence of the **3' poly A tracks**.

Therefore we can use poly dT as a single primer for a variety of different eukaryotic mRNA's.

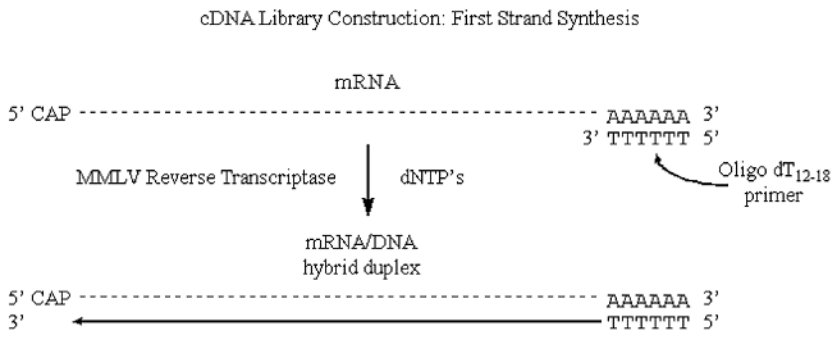


Figure 3.6.1: *First strand synthesis*

Note that we have produced ***complementary DNA (or cDNA)*** *to the original mRNA strand.*

If we can introduce "nicks" into the RNA half of this DNA/RNA duplex then the situation would be very similar to that observed in "lagging strand" synthesis of prokaryotic genomic DNA.

- Nicks in the RNA half of the molecule can be introduced via the action of the enzyme RNAse H.
- This enzyme exhibits endonucleolytic cleavage of the RNA moiety of RNA/DNA hybrids, as well as 5'->3' and 3'->5' exoribonuclease activity.
- In other words, it will nick the RNA and then proceed to digest back in both directions:

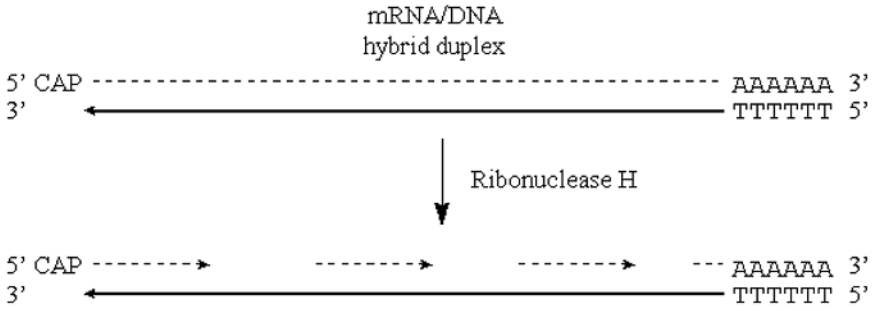


Figure 3.6.2: *Nicks in mRNA*

- These RNA fragments can now serve as primers for DNA synthesis by *E. coli* Pol I. This enzyme will also translate the "nicks" to effectively remove the RNA primers:

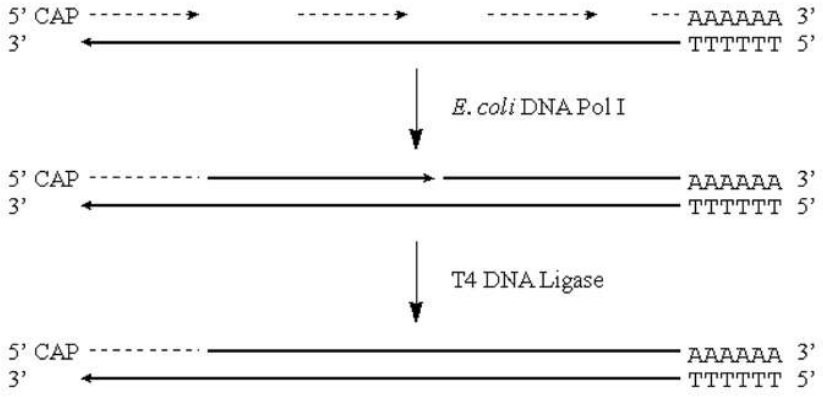


Figure 3.6.3: *DNA synthesis*

Note that we will potentially have either a residual 5' RNA cap region, or a gap at the 5' end of the original mRNA strand.

Insertion of cDNA into plasmid.

To complete our construction of a useful cDNA library we need a way to *maintain and propagate* our cDNA.

- We can accomplish this by inserting the cDNA into an appropriate **plasmid**.
- There are two classical ways of accomplishing this feat:

1. Homopolymeric tailing
2. Linker addition

Homopolymeric tailing

Terminal transferase is an unusual DNA polymerase found only in a type of eukaryotic cell called a *prelymphocyte*.

- In the presence of a divalent cation the enzyme catalyzes the addition of dNTP's to the 3'-hydroxyl termini of DNA.
- When the nucleotide to be added is a purine, Mg^{2+} is the cation used.
- When the nucleotide to be added is a pyrimidine, Co^{2+} is used.
- Depending on the reaction conditions, anywhere from three to several thousand bases will be added.

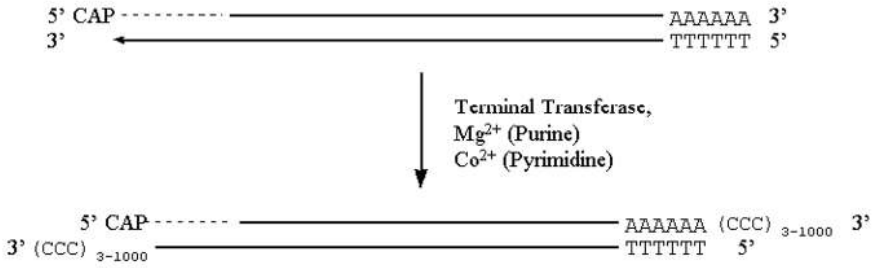


Figure 3.6.4: *Terminal transferase activity*

How can we insert this into a plasmid?

- If we cut our plasmid and also treat it with terminal transferase, except now we add the *complementary base* to the one we added to our cDNA, we can anneal and ligate the cDNA into the plasmid.

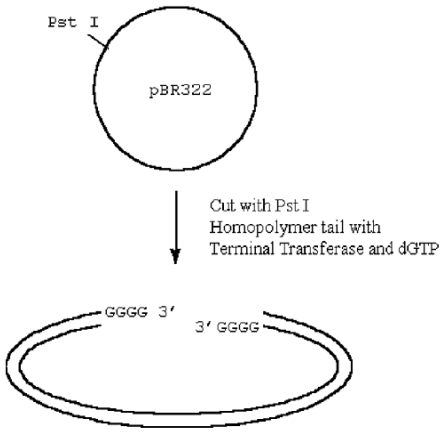


Figure 3.6.5: *Ligating cDNA into the plasmid*

- The utility of inserting the C-tailed cDNA insert into a G-tailed Pst I site in the vector is as follows:

1. The Pst I recognition sequence and cleavage site is
   5' C T G C A G 3'
   3' G A C G T C 5'
2. Cleavage of this site by Pst I, followed by G-tailing will produce
   5' C T G C A $(G)_n$ G 3'
   3' G $(G)_n$ A C G T C 5'

Thus, the Pst I recognition cleavage site is regenerated and the C-tailed cDNA insert can be excised again from the library vector by cleaving with Pst I.

Linkers

An alternate method to insert cDNA fragments into a library vector is through the addition of "linkers".

- Linkers are short oligonucleotides (~18 to 24 mers) which are typically *palindromic* and contain a single or repeated restriction *endonuclease recognition sequence*.
- The palindromic nature allows the linker oligonucleotide to *self-hybridize* to form a blunt ended *duplex*.
- If the ends of the cDNA fragments are blunt, then *the linker can be ligated to both ends to introduce useful terminal restriction sites*.

The steps in linker addition are as follows:

1. Treatment of cDNA with S1 nuclease (to remove possible 5' cap mRNA fragment remaining in cDNA duplex
2. Convert potential "ragged" ends to blunt by treatment with Pol I (will fill in 5' overhangs and chew back 3' overhangs)
3. Methylate cDNA at potential internal Eco RI sites by treatment with Eco RI methylase (plus S-adenosyl methionine)
4. Ligate linkers to blunt, methylated cDNA using T4 DNA ligase
5. Cut linkers with Eco RI restriction endonuclease
6. Remove linker fragments from cDNA fragments by agarose gel electrophoresis
7. Ligate cDNA to vector DNA fragment (opened up by Eco RI restriction endonuclease

Period of time between first man-powered flight and landing on the moon (1902-1969):
67 years
Period of time between discovery of structure of DNA and determination of the sequence of the entire human genome (1953-2010?)
57 years (?)

Editorial Note

This textbook was published in 1998. The Human Genome Project was completed in 2003.

Genomic DNA libraries

Size of some genomes and chromosomes:

Comparative Sequence Sizes	(Bases)
(yeast chromosome 3)	350 Thousand
Escherichia coli (bacterium) genome	4.6 Million
Largest yeast chromosome now mapped	5.8 Million
Entire yeast genome (completed 5/96)	15 Million
Smallest human chromosome (Y)	50 Million
Largest human chromosome (1)	250 Million
Entire human genome	3 Billion

- The human genome contains approximately 50,000 unique genes within 3-4 billion base pairs of DNA, scattered about in 23 pairs of chromosomes.

Fragmentation of genomic DNA for library construction

Restriction endonuclease digestion

- A six-cutter (e.g. Eco RI) will cut on average *every 4.1 Kb*. Complete digestion of human DNA with this type of enzyme will result in approximately 1 x 10^6 unique fragments.
- What is the probability of finding a clone within a given library?

The exact probability of having any given DNA sequence in the library can be calculated from the equation

$$N = \ln(1 - P)/\ln(1 - f)$$

P is the desired probability
f is the fractional proportion of the genome in a single recombinant
N is the necessary number of recombinants

For example, how large a library (i.e. how many clones) would you need in order to have a 99% probability of finding a desired sequence represented in a library created by digestion with a 6-cutter?

$$N = \ln(1 - 0.99)/\ln(1 - (4096/3x10^9))$$
$$N = 3.37 \times 10^6 \text{ clones}$$

Thus, from this type of analysis we can see that we need a technology which will allow us to achieve the following:

1. *Stable insertion of relatively large DNA fragments into our cloning vector*
2. *High efficiency of insertion and the ability to handle large numbers of clones*

- For example, when plating *E. coli* colonies on a 3" petri plate, the maximum practical density to allow isolation of individual colonies is about 100-200 colonies per plate.
- If we were to try to plate our library of 3.37×10^6 in such a way would need about 22,500 plates.
- Not only that, but such large DNA fragments are not well tolerated in typical *E. coli* cloning vectors such as pBR322.

Bacteriophage lambda vectors are commonly used for construction of genomic libraries

Bacteriophage l is an *E. coli* phage with a type of icosahedral phage particle which contains the viral genome:

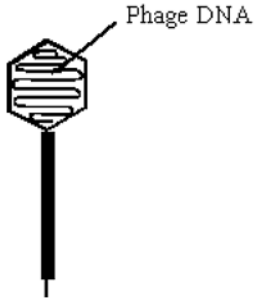


Figure 3.6.6: *Bacteriophase* l

- During replication, the phage DNA is produced in a concatameric form, which is cleaved by appropriate endonucleases to allow packaging of a single genome within the phage capsid.
- It was found that internal regions of the phage genome, which were not essential to phage replication, could be removed and replaced with DNA of interest.
- This hybrid DNA could be efficiently packaged, and form an infective phage.

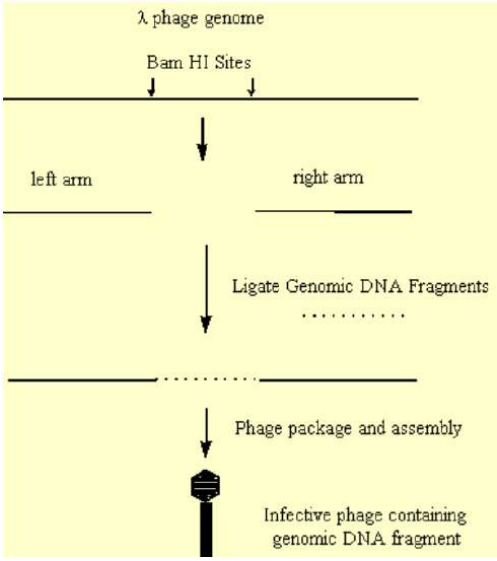


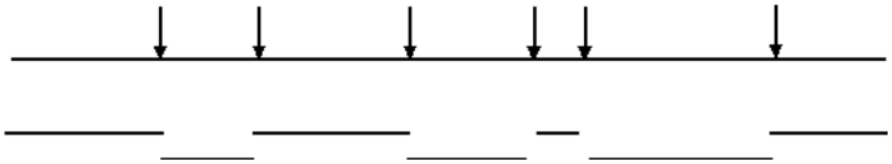
Figure 3.6.7: *Creation of ineffective phage*

The advantages of this type of system vs plasmids like pBR322 are:

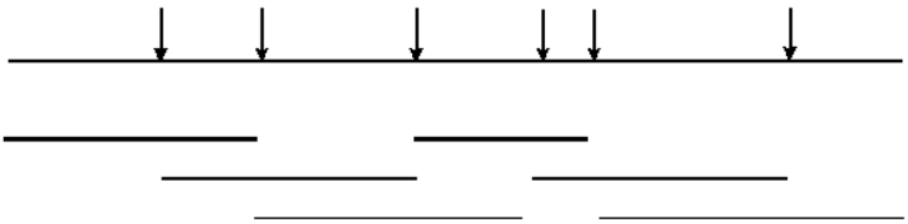
1. *The phage genome is able to package efficiently with DNA inserts as large as 20 Kb.*
2. *Furthermore, the packaged phage are highly infectious and infect E. coli at a much higher efficiency than plasmid transformation methods.*

Incomplete Digestion of Genomic DNA will allow identification of sequence overlaps

Complete digestion with an endonuclease will result in a library containing *no overlapping fragments*:



However, *incomplete digestion* will result in a library containing overlapping fragments:



- Thus, the sequence information obtained from *one clone* will allow the isolation of clones containing *neighboring (overlapping) sequence information.*
- This can allow large contiguous stretches of sequence information to be obtained ("Chromosome Walking").

Probing libraries

Once a library (cDNA or genomic) has been constructed we want to be able to identify clones which contain DNA of interest.

- For example, from protein sequence information we can *deduce possible stretches of the corresponding DNA sequence* (there will however be ambiguity due to the degeneracy of codons).
- If we can synthesize an oligonucleotide complementary to our DNA sequence of interest we can use it to specifically hybridize to the appropriate clone in our libraray (i.e. to **probe** our library).

In standard methodologies the oligonucleotide is *phosphorylated* at the 5' end with radiolabeled g^{32}P-ATP and *T4 polynucleotide kinase*.

- The probe is then incubated with individual phage plaques which have been fixed onto nitrocellulose and their DNA denatured by treatment with base.
- If the plaque contains complementary DNA to to probe sequence, the probe will hybridize.
- If the nitrocellulose (containing many individual plaques) is exposed to x-ray film, *only those plaques with hybridized probe will show up (as a dark spot)*:

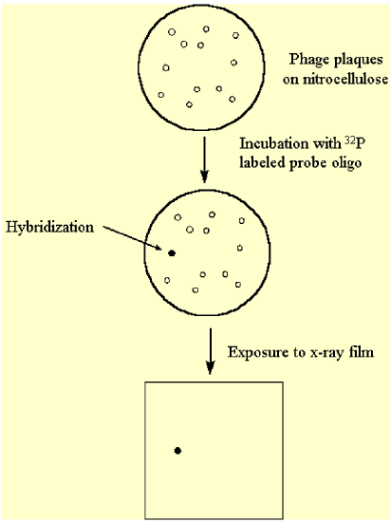


Figure 3.6.8: *Radiolabeled plaque*

Note that its important to keep track of the orientation of the nitrocellulose in relationship to the x-ray film (usually radioactive ink is used to identify the nitrocellulose orientation).

False positives

If we are designing DNA probes from protein sequence information we will have *possible ambiguity* in our deduced DNA sequence used for the design of the probe.

- Usually 14-24mer oligonucleotides are used as probes, a 14-24mer probe means we need a stretch of *5-8 amino acids* in the polypeptide.
- Given the choice, the best amino acid sequences to look for in a polypeptide are those with *low codon degeneracy* (see above).
- Thus, we would look for a short stretch of polypeptide sequence hopefully containing*Met or Trp*, and with the remaining amino acids comprising either*Phe, Tyr, His, Gln, Asn , Lys, Asp, Glu or Cys*.
- Regions including *Leu, Arg or Ser* are to be avoided (6 codons each).

During oligonucleotide synthesis multiple bases will be incorporated at ambiguous positions.

- Thus our probe will actually be a *mixture of oligonucleotides*.
- The higher the degeneracy, the greater the posibility of "false positives", i.e. clones which hybridize but are unrelated to the actual sequence we want.
- Positive clones are sequenced and the deduced amino acid sequence is compared to our polypeptide sequence information to identify correct clones.

Antibodies (Immunoglobulins)

If the particular vector, or phage, used to construct a cDNA library contains a promoter region upstream of the insertion site we may be able to screen for desired clones by looking for *expression of the protein of interest*.

- In this case, we need an assay which is both *sensitive* (we will not be producing a lot of protein) and *specific* (we want to minimize any false positives).
- One of the best assays, which is both sensitive and specific, makes use of **antibodies**.

Antigen, antibody, epitope

One of the defense mechanisms of vertebrates is the ability to distinguish between **self** and **non-self** molecules.

- Thus, if a foreign molecule (either from another species or sometimes from another individual within a species) invades a vertebrate organism, the immune system functions to learn to identify that molecule.
- In future invasions by the same molecule, the organism mounts a defense against it by producing specific **antibodies** which recognize and bind to the foreign **antigen**.
- When antibodies bind to antigen certain white blood cells (macrophages and monocytes) recognize the invading body as foreign and respond by destroying it.

Antibodies are 'Y' shaped molecules which contain two identical heavy chains, and two identical light chains.

- The stem of the 'Y' comprises the *Fc (constant) domain*, and the 'arms' of the 'Y' comprise the *Fab (variable) domains*.
- Antigens bind to the *complementarity-determining regions (CDR's)* located at the ends of the Fab domains.

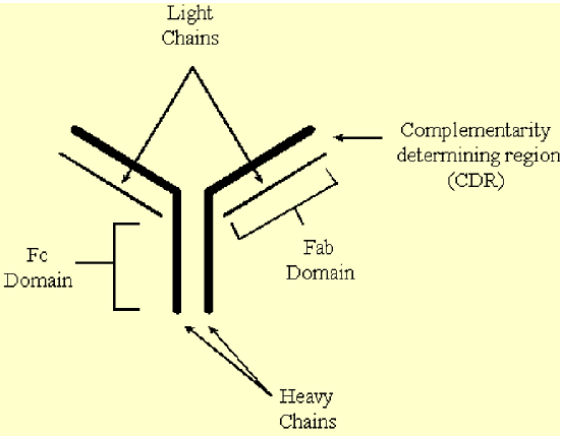


Figure 3.6.9: *Antibody structure*

Antibodies are synthesized by B lymphocytes. Each B lymphocyte is capable of producing a single type of antibody directed against a specific structural determinant, or **epitope**, on an antigen.

- Thus, an immune response to a protein antigen may result in a population of B lymphocytes each producing antibodies which recognize a different structural determinant of the foreign protein.
- An epitope may be a *contiguous region of 5 or 6 amino acids* in the foreign polypeptide, or the epitope may comprise a half dozen or so amino acids brought in juxtaposition in the native protein, yet *widely spaced in the polypeptide sequence*.
- Thus, some antibodies will recognize native *and* denatured forms of a foreign protein equally well, while other antibodies may only recognize one or the other.

If the protein of interest has been purified it can be used to *induce an immune response in a host animal*.

- Typical host animals include mouse, chicken, rabbit, goat, sheep, horse and occasionally, human.
- After an initial immunization, followed by one or more booster shots, the B lymphocytes of the host animal may produce antibodies directed against the antigen.
- The antibodies can be be purified from blood samples withdrawn from the animal. Such preparations of antibodies are said to be **polyclonal**.
- This refers to the fact that the antibodies present are from a collection of different B lymphocytes and thus will recognize a *variety of different epitopes* on the antigen protein.
- The ability to isolate antibodies from blood samples means that the host animal does not need to be destroyed.
- Of course, the size of the animal determines how much antibodies one can obtain. For example, a rabbit can provide 5 mls of blood every two weeks, a mouse provides significantly less, while a horse can provide quite a bit more.

An antibodiy isolated from a *single B lymphocyte cell population* is termed **monoclonal**.

- It recognizes a *single epitope* on the antigenic protein.

- Antibody producing B lymphocytes can be isolated from the spleen or from lymph nodes. However, they have a *finite life span*in culture, i.e. they will undergo a certain number of cell divisions and then die.
- These cells can, however, be fused with immortal (cancerous myeloma) lymphocytes to produce a **hybridoma** cell.
- Such a cell is *immortal* like the myeloma, and produces a specific antibody from the B lymphocyte. The ability to grow indefinitely in culture allows the isolation of useful amounts of specific **monoclonal antibodies**.

Sometimes immunizing with the protein of interest is problematic: appropriate amounts of purified material cannot be produced, or the protein is itself toxic at the dosage level necessary to produce an immune response.

- If partial sequence information is known, then large amounts of polypeptides representing short fragments of the protein, can be synthesized and used to immunize the animal.
- Often these polypeptides are covalently attached to a carrier protein (typically serum albumin) to enhance the antigenic response.
- Antibodies produced against such peptides will recognize only epitopes within the polypeptide. Thus, even polyclonal antibodies would be quite limited in their epitope recognition.

As with radiolabeled oligonucleotides, antibodies can be used to identify library clones which contain a cDNA of interest. *This method would of course rely upon a host vector or phage which contains a promoter upstream from the site of insertion of the genomic DNA.*

- Antibodies can be used to screen viral plaques or plasmid clonies which have been bound to nitrocellulose.
- Bound antibodies can be identified using radiolabeled protein A (which binds to immunoglobulins) or via a second antibody (which, like protein A, can recognize general immunoglobulins) which has a dye or dye releasing enzyme covalently attached.

This page titled 10.3.6: cDNA and Genomic Libraries is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **3.6: cDNA and Genomic Libraries** by Michael Blaber has no license indicated.

10.3.7: Protein Purification

Assays, Specific Activity, Initial Fractionation

A successful protein purification procedure can be nothing short of amazing. Whether you are starting off with a recombinant protein which is produced in *E. coli*, or trying to isolate a protein from some mammalian tissue, you are typically starting with gram quantities of a complex mixture of protein, nucleic acids, polysaccharide, etc. from which you may have to extract milligram (or microgram!) quantities of desired protein at high purity, and hopefully with high yield.

The first step in any purification is the development of a *specific assay* for the protein of interest. The specific assay can be based upon some *unique characteristic* of the protein of interest

- Enzymatic activity
- Immunological activity
- Physical characteristics (e.g. molecular mass, spectroscopic properties, etc.)
- Biological activity
- Ideally, an assay should be
  - *Specific* (you don't want a false positive)
  - *rapid* (you don't want to wait a week for the results)
  - *sensitive* (you don't want to consume all your sample in order to assay it)
  - *quantitative* (you need an accurate way to measure the quantity of your protein at each step in the purification)

Western Blotting

Antibodies can be used in a method called Western blotting, which is useful for determining levels of protein expression and for assaying proteins during purification. This method usually involves the following steps:

1. A protein sample is subjected to polyacrylamide gel electrophoresis.
2. After this the gel is placed over a sheet of nitrocellulose and the protein in the gel is electrophoretically transfered to the nitrocellulose.
3. The nitrocellulose is then soaked in gelatin to "block" its ability to non-specifically bind proteins.
4. The nitrocellulose is then incubated with the specific antibody for the protein of interest.
5. The nitrocellulose is then incubated with a second antibody which is specific for the first antibody. For example, if the first antibody was raised in rabbits, the second antibody might be termed "goat anti-rabbit immunoglobulin". What this means is that rabbit immunoglobulins were used to elicit an antibody response in goats. The goat antibodies (polyclonal) will include those which recognize the conserved region in the rabbit antibodies. Since the Fc region is conserved, it will bind to any and all rabbit antibodies, including those on the nitrocellulose paper.
6. The second antibody will typically have a covalently attached enzyme which, when provided with a chromogenic substrate, will cause a color reaction.
7. Thus the molecular weight and amount of the desired protein can be characterized from a complex mixture (e.g. crude cell extract) of other proteins.

In a variation of the above, the protein sample may be blotted directly on a nitrocellulose paper (called a *dot blot*) without first running a gel. This may be desirable if, for example, the antibody is monoclonal and recognizes an epitope which is dependent upon native structure (which would be destroyed upon running an SDS PAGE).

In addition to their varied uses, antibodies can also be used to purify proteins.

- If relatively large amounts of an antibody can be obtained, they can be covalently attached to a chromatography resin (e.g. sephadex beads).
- If a crude cell extract is run over such a column, only the protein of interest should bind, and everything else will flow through.
- The bound protein can then be eluted. This is typically achieved by moderately low pH conditions (using acetic acid). As long as the protein of interest is not irreversibly denatured by such conditions, the method will work quite well.
- One potential pitfall involves that of monoclonal antibodies being utilized to purify mutant proteins. The regions of the protein comprising the epitope cannot be modified without destroying the ability of the antibody to bind. Thus, the use of monoclonal antibodies in a purification scheme may preclude its use in purifying certain mutants.

Protein purification can be thought of as a series of *fractionation steps* designed so that:

- The protein of interest is found almost exclusively in one fraction (and with good yield)
- A significant amount of the contaminants can be found in a different fraction

During purification you will need to monitor several parameters, including:

1. *Total sample volume*
2. *Total sample protein* (can be estimated by A_{280}; 1.4 ~ 1.0 mg/ml)
3. *Units of activity of desired protein* (based on specific assay)

This basic information will allow you to keep track of the following information during each step of purification:

1. *% yield* for each purification step
2. *Specific activity* of the desired protein (units/mg total protein)
3. *Purification enhancement* of each step (e.g. "3.5x purification)

In designing a purification scheme you typically have to balance *purification* with *yield*.

- For example, it may be relatively straightforward to obtain 90% pure material with good yield.
- However, it may be difficult to improve that purity an additional few percentile with good yield.
- *The planned application of the purified protein determines the target purity*.
- If the protein is to be used to determine amino acid sequence information, maybe 90% is acceptable. However, if the material is to be used in clinical trials, 99.99+% may be the target purity.

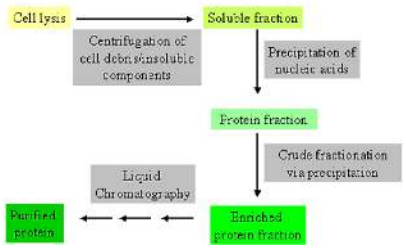


Figure 4.1.1: *Purification steps*

- It is extremely helpful to have some information not only on the general physical and chemical characteristics of the protein you are trying to purify, but also on the *contaminating* components.
- For example, many *E. coli* proteins are generally *low molecular weight* (<50,000 Da) and somewhat *acidic* in isoelectric point

Usually the initial steps in purification make use of general physical and/or chemical differences between soluble proteins and other cell components.

- For example, soluble proteins can be separated from general cellular debris, and intact cells, by *centrifugation*.
- Thus, cells are physically disrupted (via homogenization or a cell press) to allow release of cell contents. This is then followed by centrifugation to separate generally soluble components from those which are insoluble.
- It is at this point that data collection begins in order to monitor the purification.

Nucleic acids can sometimes be readily removed from the sample by the addition of large cationic compounds such as *polyethylene imine*, or *streptomycin sulfate*.

- The nucleic acids bind to these compounds via electrostatic interactions and the complex precipitates and can be removed via centrifugation.
- The same general result can be obtained by mixing in ion exchange resins which are *anion exchangers* (i.e. the resins contain cationic groups) and then filtering or centrifuging to remove. As with either method, it should be confirmed that the desired protein is *not* bound as well.

Crude fractionations of proteins can be achieved by adding various quantitites of precipitants such as *ammonium sulfate*, or *polyethylene glycol* (PEG).

- For this type of purification step an initial experiment is performed to monitor the fraction of overall protein, as well as desired protein, remaining in solution (and pellet) as a function of precipitant concentration.

Ammonium Sulfate (% saturated)	**0**	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**
Sample A_{280}	1000	900	600	200	100	75	50	40	25	20
Activity assay(units)	200	200	200	190	170	100	30	5	0	0

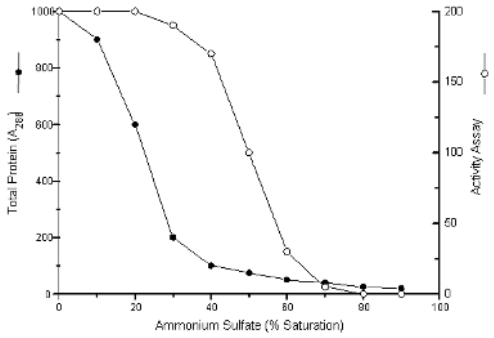


Figure 4.1.2: *Protein activity as a function of precipitant concentration*

- In this particular example we are in luck: at around 30% ammonium sulfate we can precipitate about 80% of the total protein concentration in our sample, yet our activity assay for our desired protein indicates that about 95% of our desired protein is still soluble.
- At 80% ammonium sulfate all of our desired protein has precipitated. Thus, from these results we would do the following:

1. Add ammonium sulfate to our sample to a concentration of 30% saturation
2. Centrifuge and discard the pellet
3. Add ammonium sulfate to 80% saturation
4. Centrifuge and keep the pellet. Resuspend the pellet in buffer to solubilize the protein.

- We would expect about a 5-fold purification with about 95% yield.

Column Chromatography - Ion exchange; Dialysis and Concentration

Column chromatography

After initial fractionation steps the typical procedure is to move to *column chromatography*.

- In column chromatography we have a glass tube (column) which is filled with a material ("resin") which has certain physical/chemical characteristics.
- These characteristics allow it to interact in various ways with different proteins.
- Some common types of chromatographic resins include:

1. Ion exchange
2. Hydrophobic
3. Gel filtration
4. Affinity

Ion exchange

Ion exchange resins contain *charged groups*.

- These may be *acidic* in nature (in which case the resin is a *cation* exchanger)
- or *basic* (in which case it is an *anion* exchanger).
- Cation and anion exchangers may be broken down further into *weak* and *strong* exchangers (reflecting binding affinity).

Type of exchanger	Functional group	Common name
Weak cation exchanger	carboxymethyl	CM cellulose/sephadex
Strong cation exchanger	sulfopropyl	SP sephadex
Weak anion exchanger	diethylaminoethyl	DE cellulose/sephadex
Strong anion exchanger	quaternary amine	QAE sephadex

- Usually, samples are loaded under *low ionic strength* conditions and bound material is eluted using either a step or gradient elution of buffer with *higher ionic strength*.
- Generally speaking, a protein will bind to a cation exchange resin if the buffer pH is *lower than the isoelectric point (pI)* of the protein, and will bind to an anion exchange resin if the pH is *higher than the pI*.

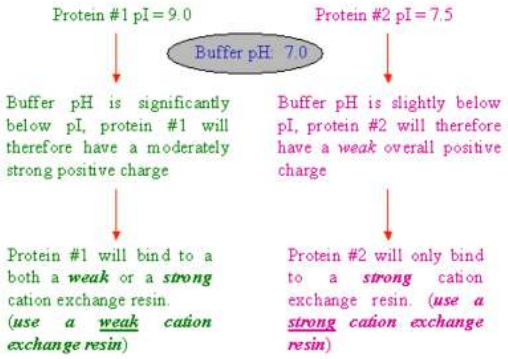


Figure 4.1.3: *Protein binding to resins*

- Knowledge of the pI of the protein is therefore helpful in designing a purification protocol using ion exchange resins (however, you can always simply try different resins to see which works best).

Generally speaking, ion exchange columns are short and fat in dimensions.

Elution of proteins from ion exchange resins

- Proteins bound to ion exchange resins are bound via non-covalent ionic (salt-bridge) interactions. We can compete for these ionic binding sites on the resin with other ionic groups, namely, ***salts***
- There are two general types of methods when eluting with a salt solution: 1. **Gradient** elution and 2. **Step** elution
- A gradient elution refers to a smooth transition of salt concentration (from low to high) in the elution buffer. Weakly binding proteins elute first, and stronger binding proteins elute last (i.e. they require higher salt concentrations in the buffer to compete them off the column)
- A gradient salt concentration can be made using a **gradient maker**. In its simplest form, this consists of two containers (***must be the same shape***) connected by a siphon (or tube at the bottom). One container contains the low salt buffer, and the other contains high salt buffer. The buffer is withdrawn from the low salt container:

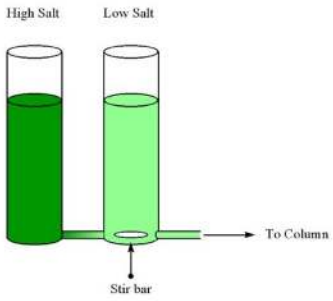


Figure 4.1.4: *Gradient maker*

- This will produce a linear gradient from low to high salt concentrations over the total volume of the gradient

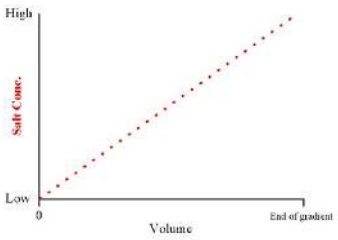


Figure 4.1.5: *Salt concentration and volume*

- If we know the concentration range of salt over which a protein of interest will elute we can simply elute with a buffer containing that concentration of salt. This is known as a **step elution**.
- Step elutions are generally faster to run, and elute the protein in a smaller overall volume than with gradient elutions. They generally work best when contaminants elute at a significantly different salt concentration than the protein of interest

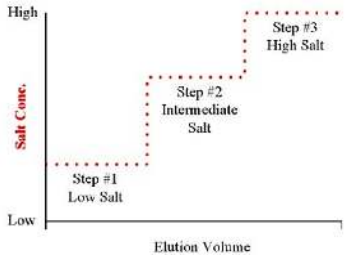


Figure 4.1.6: *Step elution*

Note that after ion exchange chromatography the protein of interest will be in a buffer with a potentially high salt concentration. This must be taken into account before proceeding with the next step in the purification scheme

Dialysis

- After an ammonium sulfate precipitation step, or an ion exchange chromatography step, the protein of interest may be in a high salt buffer. This may be undesirable for several reasons. How do we get rid of salt in our sample?
- One of the most common methods is that of **dialysis**
- The method of dialysis makes use of **semi-permeable membranes**. In the simplest example, this membrane is manufactured in the form of tubing (looking much like a sausage casing)
- The main feature of this membrane is that it is porous. However, the pore size is such that while small salt ions can freely pass through the membrane, larger protein molecules cannot (i.e. they are retained). Thus, dialysis membranes are characterized by the molecular mass of the smallest typical globular protein which it will retain.
- This is commonly referred to as the **cutoff** of the tubing (e.g. Spectrapore #6 dialysis tubing has a cutoff of 1,000 Daltons, meaning that a 1,000 Dalton protein will be retained by the tubing but that smaller molecular mass solutes will pass through the tubing)
- Dialysis proceeds by placing a high salt sample in dialysis tubing (i.e. the dialysis "bag") and putting it into the desired low salt buffer:

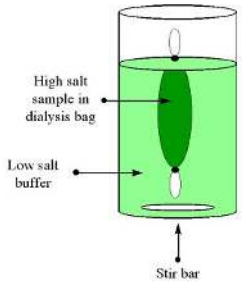


Figure 4.1.7: *Dialysis*

- Over time the concentration of low molecular mass solutes within the bag, and in the low salt buffer, will come achieve equilibrium. In practical terms (for the above case) salt molecules will diffuse out of the bag into the low salt buffer:

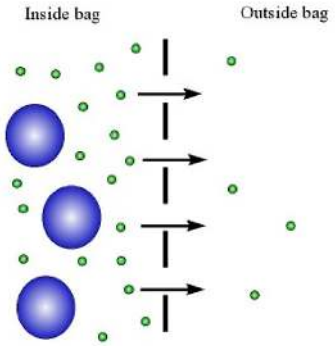


Figure 4.1.8: *Salt diffusion*

- At equilibrium the salt concentration of the sample can be calculated as follows:

$$\frac{(\textit{sample volume}) \times (\textit{sample salt concentration}) + (\textit{buffer volume}) \times (\textit{buffer salt concentration})}{\textit{total volume}} = \textit{final salt concentration} \quad (10.3.7.1)$$

Note

Often the buffer salt concentration is 0 M

- The buffer volume for the dialysis is a function of the required final concentration of salt in the sample

Example 4.1.1:

Dialysis example

We have a 10ml protein sample from an ion exchange column elution pool which contains 1.0M NaCl. For our next step in the purification we can have no more than 1mM NaCl in the sample.

$$\frac{(\text{sample vol})*(\text{sample salt conc}) + (\text{buffer vol})*(\text{buffer salt conc})}{\text{total vol}} = \text{final salt conc}$$

$$\frac{(0.01\text{L})*(1.0\text{M}) + (\text{buffer vol})*(0)}{\text{total vol}} = 0.001\text{M}$$

$$\frac{(0.01\text{L})*(1.0\text{M})}{0.001\text{ M}} = \text{total vol}$$

$$10\text{ L} = \text{total vol}$$

Therefore, the required buffer volume would be (total vol - sample vol) = 9.990 L (or ~ 10 L)

- Thus, if we dialyzed 10mls of sample (with 1.0M NaCl conc) in 10 L of water after equilibrium the NaCl concentration in the sample would be 1.0 mM.

- Note that in the above example this would commonly be referred to as a "1:1,000" dialysis.
- Suppose that we don't want to make up 10 L of buffer? We can actually achieve the same results with two sequential "1:32" dialyses (i.e. the square root of the 1:1,000 dialysis - in other words, two sequential 1:32 dialyses is equivalent to a single 1:1,000 dialysis):

First dialysis versus 310 ml of buffer: sample NaCl conc will be (10*1.0)/(320) = 31 mM

Second dialysis versus 310 ml of buffer: sample NaCl conc will be (10*0.031)/(320) = 0.97 mM

Thus, instead of making 10 L of buffer, we could make only 620 ml and achieve the same results with two dialysis steps

- In this case, removing the salt would take twice as long, i.e. we need to perform two dialysis steps. How long does dialysis take?

A useful rule of thumb is that for most types of dialysis tubing the dialysis is 80% compete after four hours

- One consequence of dialysis to watch out for is that while salt ions are moving out of the bag, water molecules are moving into the bag. Thus the volume of sample may actually increase (the bag will swell) and, therefore, the protein concentration will decrease
- In the extreme case, the bag may actually swell to the point of rupture. Therefore, *it is a good idea not to fill the bag completely, but leave a void to allow for potential swelling*.

Concentration

- What if our protein sample is too dilute for our needs? How can we concentrate our samples?
- One common method is, again, to use a semi-permeable membrane for this purpose.
- A very simple method is to place our sample in a dialysis bag and coat it with a high molecular weight solute which can readily be dissolved by the buffer.
- For example, polyethylene glycols and polyvinyl pyrolidones can have very large molecular masses (i.e. 20,000 Da). These compounds are also readily dissolved in water. If our sample in a dialysis bag is coated with dry forms of the above polymers, water will leave the dialysis bag (it can go through the pores) and hydrate the polymers. The result is a decrease in volume of buffer in the dialysis bag (the protein will be concentrated).
- In another variation, the semi-permeable membrane is manufactured into a flat disk and placed at the bottom of a container which holds our sample. In one method the container is pressurized and forces buffer out of the container (protein is retained and is concentrated). In another method, the vessel is centrifuged and the centripetal force achieves the same goal as pressure in the previous example.

For both dialysis and concentration, it is essential that the membrane does not interact with the protein (i.e. has no affinity for, and will not bind, the protein)

- With the pressure type concentrators, ***dialysis and concentration can be achieved in tandem***. For example, the sample can be concentrated and then buffer added to the sample. The sample is then concentrated again. Every time buffer is added the salt concentration is reduced. After repeated cycles of this, the salt concentration is at the desired level and the sample is concentrated to the desired final volume.

Gel Filtration, Affinity and Hydrophobic resins; Preparation of Resin, Plumbing

Gel filtration

Gel filtration does not rely on any chemical interaction with the protein, rather it is based on a **physical** property of the protein - that being the *effective molecular radius* (which relates to mass for most typical globular proteins).

- Gel filtration resin can be thought of as beads which contain pores of a defined size range.
- Large proteins which cannot enter these pores pass around the *outside* of the beads.
- Smaller proteins which can enter the pores of the beads have a longer, tortuous path before they exit the bead.
- Thus, a sample of proteins passing through a gel filtration column will *separate based on molecular size*: the big ones will elute first and the smallest ones will elute last (and "middle" sized proteins will elute in the middle).

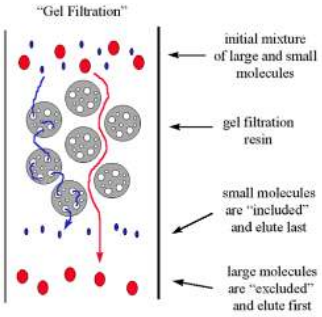


Figure 4.1.9: *Gel filtration*

- If your protein is unusually "small" or "large"*in comparison to contaminating proteins* then gel filtration may work quite well.

Where will a protein elute in a gel filtration experiment?

- There are two extremes in the separation profile of a gel filtration column.
- There is a critical molecular mass (large mass) which will be ***completely excluded*** from the gel filtration beads. All solutes in the sample which are equal to, or larger, than this critical size will behave identically: they will all eluted in the excluded volume of the column
- There is a critical molecular mass (small mass) which will be ***completely included*** within the pores of the gel filtration beads. All solutes in the sample which are equal to, or smaller, than this critical size will behave identically: they will all eluted in the included volume of the column
- Solutes between these two ranges of molecular mass will elute between the excluded and included volumes

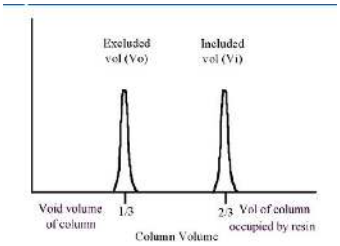


Figure 4.1.10: *Excluded vs. included volume*

As a general rule of thumb, the excluded volume (Vo) is approximately equal to one third of the column volume, the included volume is approximately equal to two thirds of the column volume

- In gel filtration the resolution is a function of column length (the longer the better)
- However, one drawback is related to the maximum sample volume which can be loaded. The larger the volume of sample loaded, the more the overlap between separated peaks. Generally speaking, the sample size one can load is limited to about *3-5% of the total column volume*.
- Thus, gel filtration is best saved for *the end stages of a purification* ,when the sample can be readily concentrated to a small volume.
- Gel filtration can also be used to remove salts from the sample, due to its ability to separate "small" from "large" components.
- Finally, gel filtration can be among the most "gentle" purification methods due to the lack of chemical interaction with the resin.

Affinity chromatography

Affinity chromatography is a general term which applies to a wide range of chromatographic media. It can be basically thought of as some inert resin to which has been attached some compound which has a *specific affinity* for your protein of interest.

- Thus, a specific antibody attached to an inert resin would be a type of affinity chromatography.
- Other examples might include: a protease inhibitor attached to some matrix, designed to bind a specific protease
- a cofactor bound to some matrix, designed to bind to a particular enzyme
- a metal ion bound to a matrix, designed to chelate a protein with a metal binding site, and so on.

In each case, the type of resins used and the method of attachment may vary, as will the method of elution. One generalization regarding method of elution is that the bound ligand can be competed off of the column's functional group by including in the elution buffer a high concentration of the free functional group. For example, if the functional group of the column is a cofactor, then the bound protein can be competed off the column by passing a buffer containing a high concentration of cofactor (or cofactor analog) through the column.

Other methods of elution include changing the buffer conditions such that the protein is no longer in the native state (since it is the native state which confers the structure required for the specific binding interaction). This can be achieved by changing pH or by adding denaturing agents such as urea or guanidine.

With affinity chromatography, typically the purification achieved in a single step can be dramatic - on the order of several thousand fold. Single step purifications with specific affinity columns are not unheard - in fact it is an ideal goal of purification - *a matrix which recognizes only the protein of interest and none other*.

Hydrophobic resins

Hydrophobic resins contain a non-polar functional group, such as an alkane or aromatic group.

- Many proteins are able to sequester such groups on their surface and this exclusion from solvent provides the basis of the binding energy (i.e. the "hydrophobic effect").
- This interaction is enhanced by increasing ionic strength, such that proteins may *bind under high salt conditions* and *elute under low salt conditions*.
- As such these columns may be used to not only provide purification, but to *desalt* samples (for example after an initial ammonium sulfate precipitation).
- It is usually not possible to predict in advance which particular resin will bind a given protein, this is usually determined empirically. However, the longer the alkane, or the larger the aromatic compound, the stronger the binding typically will be.

Due to the nature of hydrophobic interactions and ionic strength, hydrophobic chromatography and ion exchange chromatography can be conveniently used sequentially. For example, after ion exchange the protein is in high salt conditions, thus it can be loaded directly onto a hydrophobic column. Conversely, a hydrophobic column is eluted in low salt, which is a requirement for binding to an ion exchange resin.

A distinction should be noted between hydrophobic interaction chromatorgraphy and reverse phase chromatography

- Hydrophobic interaction chromatography is performed in aqueous solvent conditions and changes in ionic strength are used to elute the column. The protein typically binds in the native state via hydrophobic groups located on the surface of the protein. The native state is retained during the elution conditions
- Reverse phase chromatography utilizes a hydrophobic solvent (typically acetonitrile) and the binding of a ligand is a function of the phase partition between the hydrophobic nature of the solvent and column functional group. Proteins are typically denatured in such solvents and bind due to the hydrophobic nature of the entire polypeptide sequence. Since the majority of hydrophobic groups are located in the core of globular proteins, the binding is related to the denaturation of the protein and the accessibility of these groups to the column functional groups. Proteins can be purified using reverse phase chromatography, but usually must be refolded in some way to regain functionality (i.e. the native state)

Preparation of resins

The steps in preparing a chromatographic resin typically involve:

1. Hydration of resin
2. Decanting fines
3. Equilibrating the resin and preparing a slurry
4. Degassing the slurry

- Resins come either dry or preswollen. If they are dry they need to be hydrated. This is usually accomplished by mixing the dry resin with buffer and letting it hydrate slowly overnight (or faster at higher temperatures
- After the resin has hydrated and settled, very fine particles will settle at the top. These "fines" slow the flow rate of the packed resin. The settled resin is therefore carefully decanted to discard these fines.
- The resin is then equilibrated in the buffer to be used for the analysis. Equilibration usually involves pH'ing the resin, or buffer exchanges. Never use a stir bar when pH'ing the resin (it can mechanically shear the resin and produce fines), rather stir the resin slurry with a stir rod.
- After the equilibrated resin has settled, an equal volume of buffer is added to produce a 50% *slurry* of resin. This is usually "thin" enough to allow air bubbles to escape when packing the column.
- Finally, the slurry is degassed prior to packing the column. This will help minimize the formation of air bubbles.

Packing the column

Low pressure columns are typically packed using gravity.

- Add a small amount of buffer to the bottom of the column.
- Place a *packing reservoir* on the top of the column. Since we will be using a 50% slurry we will have a volume which is 2x the column volume and its best to pour the resin in all at one time. Thus, the packing reservoir should have a volume equal to, or greater, than the column volume.
- Carefully pour the resin slurry into the packing reservoir/column, avoiding the introduction of air bubbles as much as possible
- Let the column sit for about 5 minutes to allow large air bubbles to escape
- Open the column valve at the bottom and allow the column to pack under gravity
- Note the top of the resin bed. It will move down as the column packs. When the column is packed the top of the resin bed will no longer move down.

Plumbing

Chromatography systems may be run using only gravity and a beaker to collect the appropriate fraction. Most common systems, however, will include the following:

- A pump. Usually a peristaltic pump with variable flow rate and a communications port for a controller. *The pump is usually set up to push buffer through the column, rather than sucking buffer out of the column (which can cause a low pressure condition with production of air bubbles)*
- A detector. This is typically a UV (A_{280}) detector. Most detectors are of the two-cell type - meaning that you can have plain buffer as a blank in the detector while analyzing your column fractions. The detector sends the absorbance information to a chart recorder to be displayed (printed)
- A fraction collector. This allows you to collect fractions either by number of drops (~30 per ml) or by time. In conjunction with a controllable pump, time collection translates to volume. The fraction collector will typically have an communications port to output a signal when it changes fractions and to receive commands from the detector/controller on some sophisticated systems.
- A chart recorder. This will print a continuous trace of the detector output and the fraction collector event marker (signalling when a fraction changes). Fractions can also be read individually on a UV spectrophotometer if a chart recorder is unavailable.

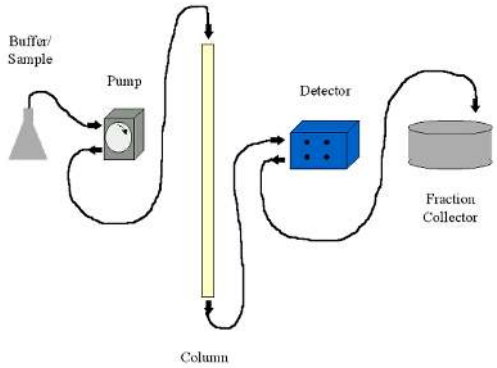


Figure 4.1.11: *Chromatography setup*

- If a gravity system is used, a safety loop should be installed to prevent the column from drying up if the buffer is used up when the column is unattended

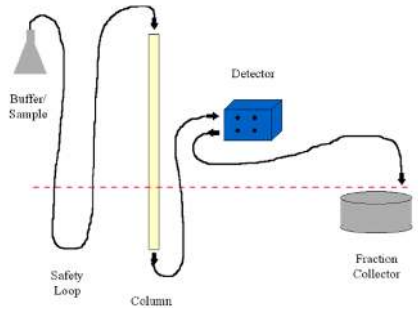


Figure 4.1.12: *Safety loop*

Note that the bottom of the safety loop is lower than the outlet to the fraction collector.

Running the Experiment, Resolving Peaks

The following represents an example of a low pressure liquid chromatography (ion exchange resin) experiment.

Sample:

- Volume = 90 mls
- A_{280} = 1.8
- Total A_{280} = 162

Column:

- DE-52 (diethylaminoethyl cellulose; anion exchange)
- Size = 1.0 x 12.7 cm
- Volume = $\pi \times (radius)^2 \times height$ = 40 mL

Fraction Collector:

- 10 mls / fraction (~300 drops/fraction)

The chromatogram for this experiment looked like this:

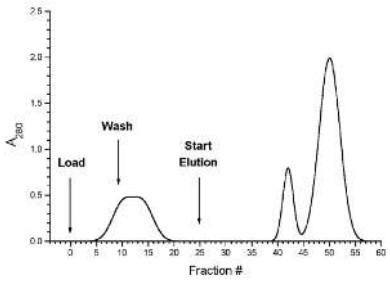


Figure 4.1.13: *Chromatogram*

The following events took place during this chromatography run:

1. Note the tick marks on the chromatogram.

- The "event" marker from the fraction collector notifies the chart recorder when a tube change takes place.
- The experiment begins with the tick next to the '0' on the x-axis ("*tick 0*"); this indicates the *start* of fraction (tube) number 1.

- The next tick mark ("tick 1") indicates the *end* of fraction number 1, and the *start* of fraction number 2.
- Thus, *fractions span the gap between the tick marks*.

2. The sample loading is begun at tick 0.

3. Sometime during fraction 5 we begin to notice the absorbence of the column effluent increasing

- It has taken about (5 fractions x 10mls per fraction) or *50 mls* from the start of loading until the detector notes any absorbance.
- This compares well with the fact that the column volume is about 40 mls and there is some volume associated with the tubing going in and out of the column.
- Thus, this 'delay' from sample load to sample detection is the *dead volume* of the system

4. Obviously, some material is not binding to the resin during the loading step. This is the *flow-through*. Is this some component of the sample which does not have affinity for the resin, or, does it represent that we have exceeded the capacity of the resin?

- If we have exceeded the capacity of the resin, then the flow-through will have an A_{280} similar to the sample being loaded
- Also, prior to exceeding the capacity, the flow-through will have some characteristic A_{280} which will then transition to another A_{280} (that of the loaded sample), resulting in a *double-plateau* chromatogram.
- In the above experiment the flow-through plateaus around A_{280}=0.5 or about 25% of the absorbance of the load. This would seem to indicate that a component, or component(s), representing one quarter of our sample, does not have affinity for the resin in the column

5. Around fraction 9 we begin to wash the column

- This makes sense because 9 fractions x 10mls per fraction = 90 mls have loaded and this is equivalent to our original sample volume (i.e. all the sample has loaded)
- The column is typically washed using the same buffer conditions in the protein sample

6. Around fraction 14 we note the A_{280} begins to decrease

- This makes sense given that we determined the dead volume of the system to be approximately 50 mls or 5 fractions. Thus, a wash which was begun at fraction 9 is observed to decrease the absorbance around fraction 14
- We continue washing the column until the A_{280} approaches 0 (*baseline*). In other words, all of the non-binding material in the sample has been washed away

7. After the A_{280} comes back down to baseline we begin our *elution* protocol. In this particular experiment we will use a linear gradient of increasing salt (NaCl) concentration (in wash buffer) to compete off the material bound to the ion exchange resin.

8. Our elution has produced two peaks: a small peak centered around fraction 42 and a larger peak centered around fraction 50

- We will have to assay each peak (and the flow through) to find out where our protein of interest has gone
- The two *elution peaks* are fairly well resolved. We could combine fractions 40-44 and call that "peak 1", and combine fractions 46-55 and call that "peak 2".

9. *Is there any material left on the column?* The integrated areas (i.e. summing the A_{280}'s of each fraction in a pool) of the flow-through, peak 1 and peak 2 are as follows

- Flow through: 4
- Peak 1: 2
- Peak 2: 10
- This gives a total integrated area of 16. Each fraction is 10 mls, so this gives a total A_{280} = 16 x 10 = 160 which is quite close to the total A_{280} of our loaded sample.
- In other words, it looks like our chromatogram is accounting for all the components in our original sample.

10. If our protein of interest was actually peak 1 (and if our yield was 100%), then this column has provided an eight fold purification (2 x 10 / 162).

Resolving peaks

- Contaminating peaks will not necessarily be completely separated from the peak which contains our protein of interest
- In the following picture there are two components being resolved, and they are present in equimolar amounts (thus, the starting purity is 50%). The yield and purity are listed for the situation where we were to pool each peak by splitting at the midpoint between them (in this particular example the yield and purity are identical in each case)

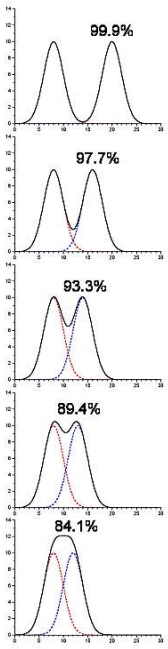


Figure 4.1.14: *Contaminating peaks*

- This gives you some idea of the amount of cross-contamination in each peak as a function of their separation from one another.
- Software to fit gaussians to a chromatogram can provide this type of information

Pooling for purity verses yield

Do we try to pool to maximize yield or to maximize purity?

Usually, you will probably be pooling fractions in such a way as to maximize the recovery of your protein of interest. However, you always have the option of pooling to increase purity, and if you have lots of protein to work with this may allow you to achieve the desired purity with fewer steps. Here's an example of how it's done:

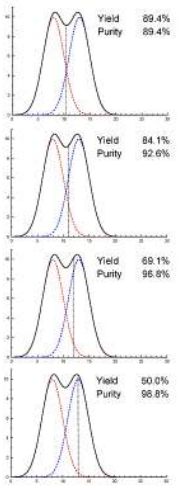


Figure 4.1.15: *Yield vs. purity*

- These are all the same chromatogram, however, we can pool them differently to get better purity (at the expense of yield
- The blue peak is the peak of interest and it is not resolved from a contaminating peak (in red).
- The vertical line represents the left-most fraction we use to pool the peak (we pool all fractions to the right of the vertical line to get our protein of interest)
- In the last panel we see that we can achieve about 98.8% purity if we are willing to part with half our protein!

Monitoring the purification

How do you know when you are finished purifying a protein?

There are several criteria. One criteria is that we cannot improve upon the *specific activity* of our sample. This value refers to the *functional activity of our sample in relationship to the total protein concentration of the sample*.

- In the initial stages of purification this value will be low (not much activity in relationship to the total amount of protein).
- This value will increase after each purification step as we *remove* other proteins from the sample.
- At some point the specific activity will *plateau*, and by definition, *if it is pure we cannot increase the specific activity*.

- There may be a published value for the specific activity which we can compare ours to.

Also, each step of the purification should be monitored by gel electrophoresis.

- In the initial stages of purification we will probably see a variety of bands, of various molecular weights, on our gel.
- After the different purification steps, we should see the disappearance of certain bands concomitant with the increasing concentration of a certain band (or bands) representing our protein.
- If we have successfully purified our protein (and if it is a single polypeptide) we should arrive at a constant specific activity and *a single band on a gel*.
- Analytical methods like HPLC or densitometer scanning of a stained gel can give us a quantitative idea of the purity of our final sample.

The following chart represents the typical data one would monitor during a purification:

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification	% Yield
Crude cell lysate	5500	6600	1.2		
30-70% Ammonium sulfate cut	1020	5910	5.8	4.8	89.5
DEAE Sephadex pool	187	5070	27.1	4.7	85.8
CM Sephadex pool	102	4420	43.3	1.6	87.2
Phenyl Sepharose pool	56	3930	70.2	1.6	88.9
Gel Filtration pool	32	2970	92.8	1.3	75.6
Affinity resin type #1 pool	5.8	2520	434.5	4.7	84.8
Affinity resin type #2 pool	5.3	2390	450.9	1.0	94.8
Total purification	**376**				
Total yield (%)	**36**				

This page titled 10.3.7: Protein Purification is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **4.1: Protein Purification** by Michael Blaber has no license indicated.

SECTION OVERVIEW

10.4: 5. Lab Notes Part 1

10.4.1: Basic practices and techniques, Laboratory notebooks, Presentation of data, Sections of a scientific report

10.4.2: Spectrophotometry

10.4.3: Quantification of Protein Concentration

10.4.4: Chromatography

10.4.5: Gel Electrophoresis of Proteins

10.4.6: Introduction to Protein Purification

This page titled 10.4: 5. Lab Notes Part 1 is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

10.4.1: Basic practices and techniques, Laboratory notebooks, Presentation of data, Sections of a scientific report

There are two main concerns in a biochemical laboratory environment, they are:

- Safety
- Accurate record keeping

Safety

A biochemical laboratory has safety concerns that include not only issues of chemical safety, but also issues of biohazard safety. In both cases, your safety depends upon a knowledge of the materials and procedures you are working with, as well as appropriate protective dress.

Safety issues will be covered in detail in the syllabus, and in the lab by the lab director, however, a few key points will be covered here:

1. Read the relevant chapter of the lab manual BEFORE arriving for the lab. To reinforce this practice, the labs will start with a short quiz related to the lab for that day. The quiz will start PROMPTLY at the beginning of class, will last approximately 10 minutes, and there will be NO MAKEUP QUIZZES for late arrivals. The beginning of lab time is used to organize the lab, and includes relevant safety information. Therefore, it is essential for all concerned that students show up promptly for the labs.

2. Dress appropriately. No open toed shoes (i.e. sandals), shorts, or bare midriffs are permitted in a laboratory setting (OSHA guidelines). YOU WILL BE ASKED TO LEAVE IF YOU ARE NOT APPROPRIATELY DRESSED. Lab coat and goggle guidelines will be addressed by the lab manager.

3. Lab attitude and behavior. Both safety and a successful learning experience are keenly associated with behavior and attitude. A cavalier attitude will result in accidents and experimental failures. Thinking about what you are doing, and asking questions, will result in success and a long life. One of the biggest problems in a laboratory setting is equipment damage due to a lack of knowledge about how to use such equipment. DO NOT USE ANY EQUIPMENT WITHOUT FIRST BEING INSTRUCTED ON HOW TO USE IT. Due to the expense of scientific equipment, labs can only function through the use of shared equipment, thus, everyone using such equipment needs to take care of it.

Accurate Record Keeping

There are typically two levels of record keeping for scientists involved in biochemical research:

1. The laboratory notebook
2. The research report

It is important to understand the purpose of each type of record keeping, and to adhere to the practices associated with each one.

The laboratory notebook

The lab notebook can be thought of as your "working record" or "experimental log". It is a sort of formalized diary, and has a specific purpose and associated formalism with regard to how to use and maintain it.

- For purposes of invention, discovery and patents, it is the primary record used to establish ownership of an invention and dates of discovery. To be useful for this legal process, certain guidelines must be followed.
- For the purpose of the scientific process it provides the necessary detail so that experiments can be repeated and the results replicated and confirmed
- For the purpose of reporting results, the lab notebook is the repository of all primary data and results

To meet the above needs, the lab notebook is:

· **bound** (no loose leaf) and

· **pages are numbered sequentially**.

· Futhermore, entries in the lab notebook are **always made in ink (black or blue)**, never pencil. If something is written that is incorrect, it can be crossed out.

In this way, an examiner of the notebook can determine whether the lab notebook is **intact** or whether pages or information have been **removed**.

Entries into the notebook for a given experiment always begin with:

· Writing the **date**

· Stating the **purpose** of the experiment

The remainder of the notebook section that covers the experiment will include any and all relevant information associated with the experiment, including:

- A description of the materials, equipment and reagents used
- A description of the methods used
- Calculations used (a truly essential aspect of trying to repeat the experiment and identifying errors)
- Tabulation of results collected
- Conclusions, hypotheses, notes, etc.

The key point is that you include enough information so that someone else can use your notebook to replicate the experiment and obtain the same results you did. In this regard, the notebook does not have to have perfect penmanship, but it must be legible and understandable by others. Diagrams are often essential. In industrial (and some academic) settings, it is a requirement that another person in the lab sign off on your notebook that they have **read, and can understand**, your notebook entry for a particular experiment (this is associated with the legal function of the notebook).

The notebook must not contain any loose bits of paper. If an experiment includes a photograph or some type of printout from an instrument, this must be firmly affixed to the notebook (i.e. glued & stapled). Any loose paper can be lost, and with it, some key information. The best approach, if you have a data printout from an instrument, is to copy it by hand into your notebook. Sometimes this is not practical, so the data must be glued & stapled. It is also not a good idea to have information beneath such added items. In some industrial settings, the lab notebooks are Xeroxed for archival purposes. In this case, Xeroxing a page will miss information if it is written underneath a photo, etc.

The first few pages of the lab notebook should be reserved for a table of contents. This is associated with the function of being able to replicate an experiment. Part of this requirement is the ability to locate a particular experiment in a given notebook. Researchers can generate dozens of notebooks involving hundreds of experiments, and a table of contents is essential. Furthermore, a researcher will often have two or more experiments on-going at one time. It is common for a notebook entry to start on one page and then reference a later page for continuation of the experiment (sort of like some magazine or newspaper articles). If appropriate, notebook pages should indicate both the referring page number, and the continuation page number.

The research report

The lab notebook is your working journal, and is not intended as a formal research report. The formal research report has **specific sections and organization**, and is a **final, polished document**relating to your research. These sections include the following (in specific order):

- Title
- Introduction
- Materials and Methods
- Results
- Discussion
- References

In many cases, such reports also have an ***abstract*** (a short, ~250 word report that precedes the Introduction and details the essential aspects and results). However, this will not be required in your own reports.

Each section of the report has a specific function:

Title

- Typically a **single sentence** that explains to the reader what the experiment is all about

Introduction

- One or more paragraphs that explain the purpose of the experiment, and provide any **relevant background information** so that the reader is prepared to follow the narrative of the report. In published research reports the introduction also lays out a logical argument as to the importance of the experiment

Materials and Methods

- A detailed "recipe", describing instrumentation, materials and supplies, and procedures used in performing the experiment. The "M&M" section is detailed enough so that the experiment can be repeated and the results confirmed by others. An essential aspect of the scientific method is that results are meaningless unless they can be confirmed by others.

Results

- Statements of fact regarding the data collected, with associated errors or deviations (e.g. all relevant values and/or numbers relating to your data). This is often represented using tables, and sometimes, figures.

Discussion

- An **interpretation** of the results, and a discussion of its meaning, including a discussion of error (where relevant). In published research reports, the discussion will also put the work in the context of other published work in the same field.

References

- If you used any texts, primary literature, or web sites in preparing your experiment or report, these should be referenced. There are a variety of formats, but the reference generally includes author, title, volume, pages and year (also publisher, if it is a text). For web sites, author and URL. A key aspect is to avoid **plagiarism**.

An addition to having the above sections, all research reports have a required **format**. The format of a report includes details of:

- Type of font, line spacing, page breaks, margins, etc.
- Page length and/or word number requirements
- Limitations on number of tables, figures, use of color, etc.
- Title page format
- Formatting of references

This course is no different, and **there is a required format for research reports**, and these can be found in the syllabus.

Common mistakes in writing the research report

Although it may seem straightforward, writing an effective research report can take years of practice. There a several aspects that can prove difficult. Here are a few things to consider:

- Do not put Results into the Materials and Methods section. The M&M section may have equations, but will not have any data listed.
- Do not put comments associated with the Discussion section into the Results section. Sometimes it is difficult to know what goes where, but here is a general guideline: information in the Results section can be thought of as statements of fact (i.e. your data), whereas, interpretation and hypotheses are part of the Discussion section. Someone might argue with your ideas in the Discussion section, but there should be no argument about information in the Results section.
- Error associated with your data is presented in the Results section as well. Do not get hung up on semantics. A "discussion of error" goes into the Results section.
- Do not duplicate statements or paragraphs in different sections. If you have said something in the Introduction, do not repeat it in the Results or Discussion section.
- Do not duplicate data. If you present data in a table, do not also present it as a figure. However, having said this, it is important to recognize that, even with scientists, people can often grasp meaning easier with a figure as opposed to looking at numbers in a table. Often, selected data is extracted and compared using an appropriate figure.

- **YOU MUST FOLLOW THE FORMATTING GUIDELINES FOR THE RESEARCH REPORT**. In the "real world" failure to follow such guidelines will result in immediate rejection of the report.

An example of a research report is **given here**.

This page titled 10.4.1: Basic practices and techniques, Laboratory notebooks, Presentation of data, Sections of a scientific report is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **5.1: Basic practices and techniques, Laboratory notebooks, Presentation of data, Sections of a scientific report** by Michael Blaber has no license indicated.

10.4.2: Spectrophotometry

The human eye responds to electromagnetic radiation within a range of wavelengths between 400-750 nm (i.e. the "visible spectrum"). Samples of light that contain a continuous spectrum of all wavelengths between 400-750nm will be perceived by the brain as "white light" (e.g. the sun). Light comprising a specific wavelength within this range is perceived by the brain as being "colored":

- ~400nm = "violet"
- ~450nm = "blue"
- ~500nm = "green"
- ~550nm = "yellow"
- ~600nm = "orange"
- ~700nm = "red"

Often objects appear colored because of their **absorption** of light within selective regions of the visible spectrum. The light from such objects that strikes our eyes (whose color we perceive) is composed of those wavelengths that the object **DID NOT** absorb.

For example, plant leaves contain two photosynthetic pigments: chlorophyll A and chlorophyll B. The chlorophyll A molecule has the ability to absorb light with a wavelength in the range of 430 and 660 nm; the chlorophyll B molecule absorbs light with a wavelength in the range of 450 and 640 nm. Thus, these two pigments in leaves absorb violet/blue and orange/red wavelengths of light (the energy that these photons represent is transferred to the chlorophyll molecules). Light with wavelengths in the range of 500-600nm is not absorbed by either molecule. Thus, after sunlight interacts with leaves the wavelengths that remain (and that our eye can perceive) are green-yellow. Therefore, plants are "green" because they don't absorb green light.

- What would an object look like if it contained a pigment that absorbed all wavelengths from 400-750nm? It would be "black"
- This is the same situation as being in a closed room with the lights turned out. **Without light there is no color**.

If we had a sample of leaf juice containing chlorophyll A and B we could quantitate the concentration of chlorophyll by the amount of violet/blue and orange/red light it absorbed. We COULD NOT quantitate it by the amount of green light transmitted (it would be essentially the same amount regardless of the amount of chlorophyll:

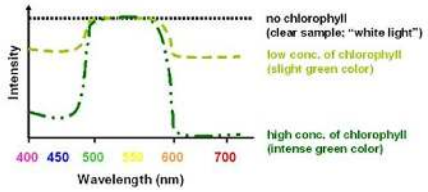


Figure 5.1.1: *Chlorophyll light transmission*

Even though the sample is "greener" with higher concentrations of chlorophyll, this is <u>not</u> due to an increase in the intensity of the green wavelength of light; it is due to a <u>reduction</u> in the violet/blue and orange/red wavelengths of light. It is this <u>reduction</u> that we can use to <u>quantitate</u> the concentration of chlorophyll. Although the sample appears green we cannot quantitate it by monitoring the green wavelength of light.

Light, Energy and Molecular Structure

Light is strange stuff, it is a radiative form of energy transfer, and in order to understand its properties we have to consider it to have both wave and particle properties. The different wavelengths of light differ in the energy they carry; the energy is directly proportional to the frequency of the light (the higher the frequency, the higher the energy):

$E \alpha n$

The energy unit is the **erg** (1Joule = 10^7ergs) and the frequency unit is **Hertz** (i.e. cycles per second, or just units of sec^{-1}). The constant of proportionality is Planck's constant, ***h***, with a value and units of **6.6×10^{-27} erg sec**

E = *h*n

erg = (erg sec)(sec^{-1})

The frequency (n) of light is inversely proportional to the wavelength (l):

n α 1/λ

Frequency has units of sec^{-1} and wavelength has units of meters. The constant of proportionality is ***c*** (the speed of light) with a value and units of 3×10^8m/sec:

n = c(1/λ) = c/λ

This relationship is true in a vacuum; however, "optically dense" materials can slow the speed of light requiring the following correction to the above equation:

n = c/*n*l

where ***n*** is the "refractive index" of the material and has a value > 1

Therefore, the energy of light is inversely proportional to the wavelength:

E = (*h*c)/(*n*l)

The "photon" is a corpuscular (particle) description of light that is the carrier of the "quanta" of energy defined in the above equation. When light is absorbed by a molecule it transfers its quanta of energy, and the photon ceases to exist. Where did the energy go?

- The atomic bonds, chemical structure and electrons of a given molecule have **specific** excited states and vibrational modes.
- These excited states and vibrational modes have defined energy levels above the ground state.
- The molecule can change from the ground state to an excited state (or vibrational mode) upon the absorption of a quanta of energy exactly equal to the difference between the ground and excited states.
- **Thus, molecules can absorb the energy associated with specific wavelengths of light, and the light is consumed in the process**.

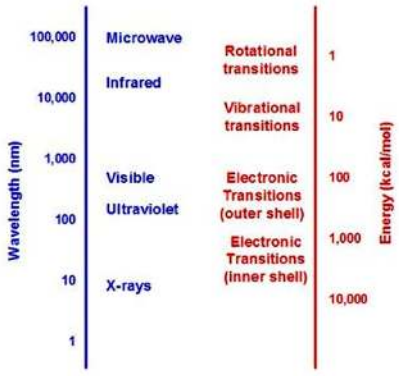


Figure 5.1.2: *Scale of light*

The atomic bonds, chemical and electronic structure of molecules are unique properties and differ from one type of molecule to another.

- Thus, the energetic differences between the ground and excited states differs from one type of molecule to another.
- Therefore, the ability to interact and absorb light, and the specific wavelengths of light absorbed differ from one type of molecule to another

The characteristic absorption pattern of the different wavelengths of light is unique for each type of molecule and is a type of **molecular "fingerprint"** that can be used to **identify and quantitate** molecules

In the example of chlorophyll given above the intensity of light passing through the sample is termed the "light **transmittance**" of the sample:

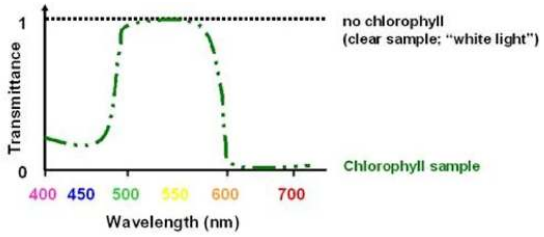


Figure 5.1.3: *Light transmittance of chlorophyll*

- Transmittance is a dimensionless number that varies from 1 (full transmittance) to 0 (no transmittance - complete absorption)

It is important to note the distinction between transmittance and absorption: they are inversely proportional

- **A sample with high absorbance has a low transmission of light**

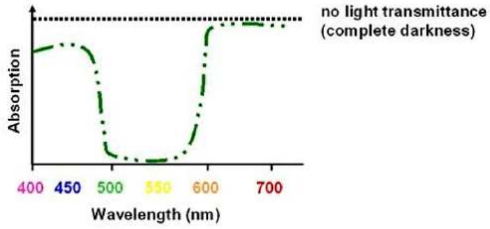


Figure 5.1.4: *Chlorophyll absorption*

How are transmittance (T) and absorption (A) quantified?

- The intensity of light shining on a sample is termed the incident light, I_0
- The intensity of light measured after passing through a sample is the transmitted light, I
- Transmittance, T, is defined as the ratio I/I_0 and will vary between 0 and 1:

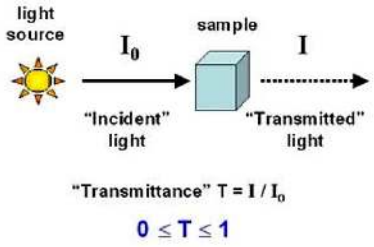


Figure 5.1.5: *Transmittance*

How is the transmittance affected by the sample concentration and physical size (i.e. path length)?

- If the transmittance is reduced due to the absorption by the sample, then the higher the concentration (***c***), the lower the transmittance. In other words, the transmittance is inversely proportional to the concentration: (Beer's law)

T α 1/*c*

- Similarly, the thicker the sample (i.e. the greater the **path length** of the light through the sample), the lower the transmittance. Thus, the transmittance is also inversely proportional to the path length (***l***): (Lambert's law)

T α 1/*l*

Thus, we expect the equation relating T to *c* and *l* to take the general form of:

T α 1/*cl*

Transmittance turns out to **decrease exponentially** with increases in the concentration and path length, thus the equation has the form:

logT α 1/*cl*

-logT α *cl*

The constant of proportionality is the extinction coefficient **e**:

-logT = ε*cl*

Since log values are dimensionless, the units of e would appear to be inverse concentration and inverse distance (e.g. $M^{-1}cm^{-1}$)

The -logTterm on the left can be written as log(1/T) and identifies the **inverse** relationship between the transmittance T and the e*cl* term. Since absorbance and transmittance are inversely related, **the e*cl* term would seem to be a convenient definition for the absorbance (A):**

log(1/T) = ε *cl* = A
(the Beer-Lambert Law)

Values for ε and *l*

- Path length ***l*** is usually in units of **cm**
- Molar extinction coefficient **ε** has units of M^{-1} cm^{-1} and is a constant of proportionality that relates the absorption of **molar** solutions
- Mass extinction coefficient $\varepsilon^{1\%}$ refers to the absorbance of a 1% by mass solution. Typically this refers to an aqueous solution that we can take to have a density of 1000g/L. A 1% by mass aqueous solution would therefore refer to the dissolution of 10g/L, or a **10mg/ml** solution of the molecule of interest.
- Since the absorbance of a molecule is a function of the wavelength (i.e. the absorption is not equal for every wavelength) the extinction coefficient must also reference a wavelength. This is typically done using a subscript:

$\varepsilon^{1\%}_{280nm}$= 14.5 g^{-1} L cm^{-1}

· In this case **a 10mg/ml solution of the molecule will have an absorbance reading of 14.5** (dimensionless units) at l = 280nm (the absorption at other wavelengths may not be known). The units of concentration are g/L, thus e will have dimensions of g^{-1} L cm^{-1}.

The relationship between changes in A, T and c

· The direct relationship between A and c means that there is a linear relationship between absorbance and concentration. If you double the concentration the absorbance will double, etc.

· The inverse log relationship between transmittance and absorbance can be stated as:

$T = 1/10^A$

- A doubling of the concentration will therefore result in a 10-fold reduction in the transmittance. Instrumentation for absorbance measurements (**spectrophotometers**) actually measure transmittance, and naturally become less accurate at low values of transmittance. Thus, the higher the absorbance reading the less accurate it is. **Most such instrumentation is inaccurate at absorbance readings > 1.5** (this works out to be at transmittance < 3% of full transmittance)

Design of Spectrophotometers

Spectrophotometers are precision instruments, however, conceptually they involve a relatively few number of parts. A simple design would look something like this:

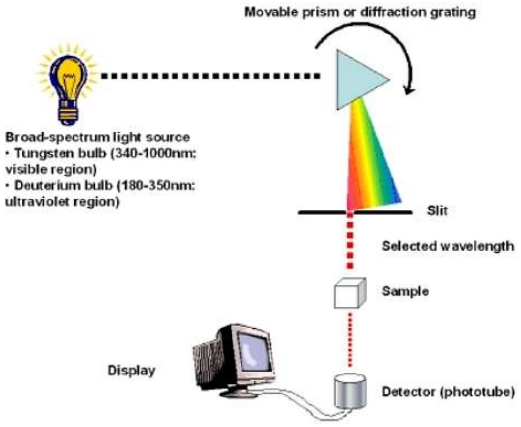


Figure 5.1.6: *Spectrophotometer*

- A tungsten lamp is used to produce wavelengths of light spanning the visible range, while a deuterium lamp is used to produce light spanning the ultraviolet range.
- The movable prism or diffraction grating is adjusted to direct the wavelength of interest towards the sample
- This is a "single beam" spectrophotometer and the reference and sample data are collected separately (the reference sample is used to determine the maximum transmission value (effectively I_0)

A modification of this design, a "dual beam" spectrophotometer, can permit simultaneous measurement of I and I_0:

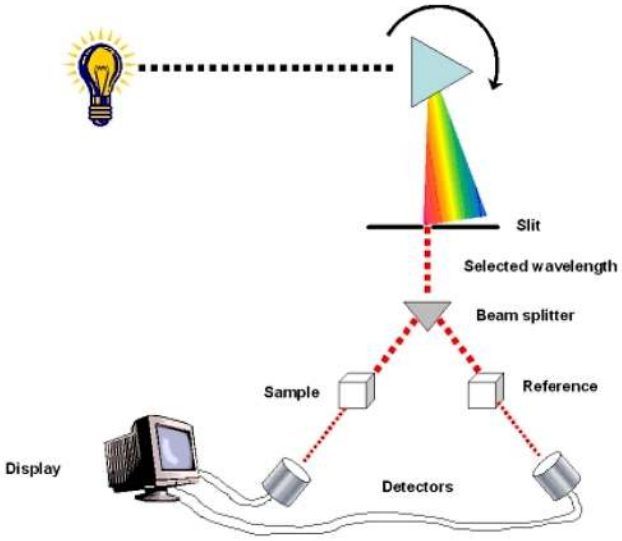


Figure 5.1.7: *Dual beam spectrometer*

Both the single and dual beam detectors allow the investigator to monitor the absorbance properties for a **single wavelength** of light (the wavelength selected by the prism/slit settings. A modification that incorporates an **array of detectors** can permit simultaneous measurement of a spectrum of wavelengths:

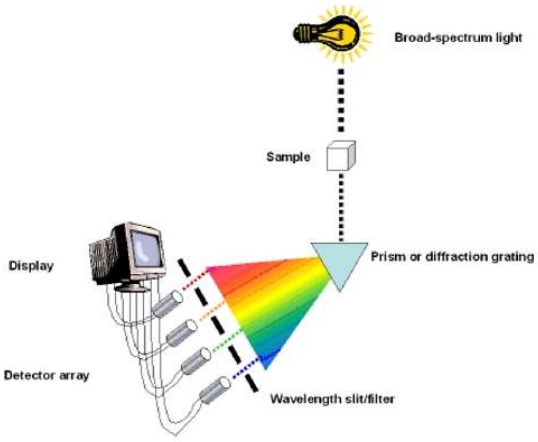


Figure 5.1.8: *Simultaneous measurement of a spectrum*

Absorbance spectra of biological molecules

Proteins

Proteins do not absorb in the visible wavelength unless they have a prosthetic group (e.g. Fe^{2+}) or an unnatural amino acid. However, the amino acids tryptophan, tyrosine and cysteine absorb light in the UV wavelength:

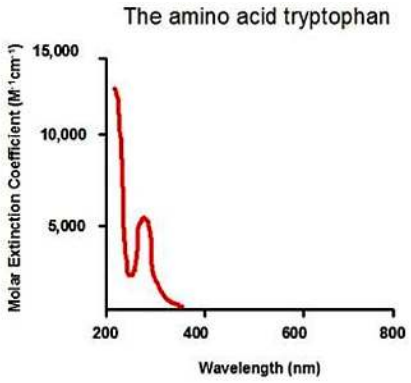


Figure 5.1.9: *Tryptophan absorption*

- Tryptophan has a peak of absorption at 280nm in the UV range
- This is a useful wavelength to quantitate the absorption of tryptophan
- Since the absorption is proportional to concentration, this is a useful way to quantitates protein concentration (for proteins containing Trp)

Nucleic acids

The aromatic rings in the bases of nucleic acids also absorb in the UV range:

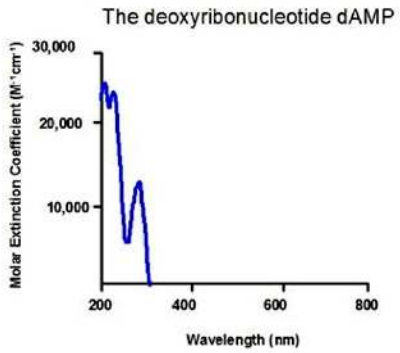


Figure 5.1.10: *Nucleic acid absorption*

- Each DNA and RNA base has a slightly different absorption spectrum
- 260 or 280nm is a typically useful wavelength to monitor concentration of nucleic acids

Note that samples of nucleic acids and proteins can both absorb at 280nm, therefore, samples of biological molecules should be **pure** in order to quantitate using UV absorption spectroscopy.

This page titled 10.4.2: Spectrophotometry is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **5.2: Spectrophotometry** by Michael Blaber has no license indicated.

10.4.3: Quantification of Protein Concentration

Review: the Beer-Lambert Law

log(1/T) = ε*cl* = A
T = 1/10^{A}

Values for ε and *l*

- Path length ***l*** is usually in units of **cm**. (note: most spectrophotometers are designed to accept 1cm wide cuvettes)
- Molar extinction coefficient **ε** has units of M^{-1} cm^{-1} and is a constant of proportionality that relates the absorption of **molar** solutions
- Mass extinction coefficient **ε $^{1\%}$** refers to the absorbance of a 1% by mass solution. Typically this refers to an aqueous solution that we can take to have a density of 1000g/L. A 1% by mass aqueous solution would therefore refer to the dissolution of 10g/L, or a **10mg/ml** solution of the molecule of interest.
- Since the absorbance of a molecule is a function of the wavelength (i.e. the absorption is not equal for every wavelength) the extinction coefficient must also reference a wavelength. This is typically done using a subscript:

ε $^{1\%}_{280nm}$= 14.5 g^{-1} L cm^{-1}

· In this case **a 10mg/ml solution of the molecule will have an absorbance reading of 14.5** (dimensionless units) at l = 280nm (the absorption at other wavelengths may not be known). The units of concentration are g/L, thus e will have dimensions of g^{-1} L cm^{-1}.

Why is it important to be able to quantitate protein concentration in a sample?

An important application of "Biotechnology" is the production of proteins as commercial products. Such products might have pharmaceutical applications (e.g. insulin, human growth hormone, tissue plasminogen activator, erythropoietin, blood clotting factor VIII.), industrial applications (e.g. subtilisin (an enzyme in detergents), 2,5-diketo-D-gluconate reductase (an enzyme in vitamin C production), as materials (e.g. silk protein in textiles, barnacle adhesion protein as a glue). In these cases, there are various aspects of successful production that require quantitation:

- How much of the protein can be produced (i.e. what is the efficiency of production)?
- How pure is the protein that is produced (industrial applications may require 90% pure, pharmaceutical applications may require 99.999% pure)

Such proteins may be isolated from natural sources (e.g. blood clotting factor VIII may be extracted from human blood), or they may be produced recombinantly (e.g. E. coli bacterial cells can be genetically engineered to produce human growth hormone). In both cases, it may be necessary to purify the protein using a series of fractionation steps. We will go into more detail about such fractionation steps in a later lecture, but the general idea is that a heterogeneous mixture of molecules can be fractionated based upon some physical property of the molecules. The following are properties that can be used to fractionate a heterogeneous mixture of biomolecules:

- Molecular mass (i.e. "big" molecules can be separated from "small" molecules)
- pKa (i.e. "acidic" molecules can be separated from "basic" molecules)
- Hydrophobicity (i.e. non-polar molecules can be separated from polar molecules)

For such fractionation steps involving proteins, we need to keep track of how much of the contaminating proteins went into one fraction and how much of our desired protein went into the other fraction. Although the details are somewhat more complicated than this simple description, **it is important to be able to quantitate protein concentration to be able to effectively purify a protein of interest**.

Once a protein is pure, it may be of considerable economic interest to be able to quantify the yield (and, therefore, be able to determine how much it cost to produce a given mass of protein). For example, the only source for human growth hormone (to treat small stature) used to be to extract it from human pituitary glands harvested from the brains of cadavers. Suffice it to say, this made the protein extremely expensive. Furthermore, the isolation from human tissues meant that the sample could also be potentially contaminated with human pathogens (hepatitis, CJD, AIDS, etc.). With the advent of genetic engineering, the production of human

growth hormone by bacterial cells (i.e. E. coli) meant that relative large quantities could be produced far cheaper (and with no threat of human pathogens).

Why not just weigh the protein?

- Most samples are typically quantities of milligrams or even micrograms, not grams, and thus, it is difficult to transfer and measure such small amounts
- Water is present in proteins, and it is extremely difficult to remove all the water (some water molecules hydrogen bond extremely tightly to proteins). Thus, the mass measurement would include some waters, and would increase the apparent mass of the protein

Absorbance spectra of biological molecules

Proteins

Proteins do not absorb in the visible wavelength unless they have a prosthetic group (e.g. Fe^{2+}) or an unnatural amino acid. However, the amino acids tryptophan, tyrosine and cysteine absorb light in the UV wavelength:

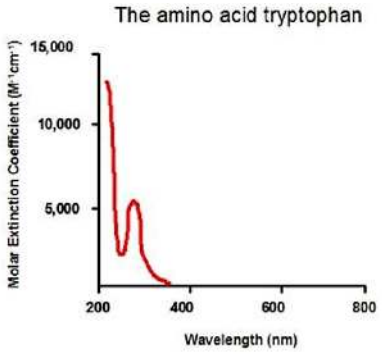


Figure 5.3.1: *Tryptophan absorption*

- Tryptophan has a peak of absorption at 280nm in the UV range
- This is a useful wavelength to quantitate the absorption of tryptophan
- Since the absorption is proportional to concentration, this is a useful way to quantitates protein concentration (for proteins containing Trp)

Nucleic acids

The aromatic rings in the bases of nucleic acids also absorb in the UV range:

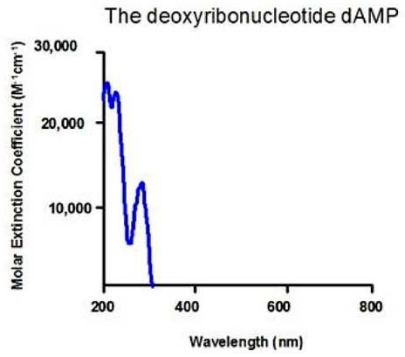


Figure 5.3.2: *dAMP Absorption*

- Each DNA and RNA base has a slightly different absorption spectrum
- 260 or 280nm is a typically useful wavelength to monitor concentration of nucleic acids

Note that samples of nucleic acids and proteins can both absorb at 280nm, therefore, samples of biological molecules should be **pure** in order to quantitate using UV absorption spectroscopy (any contaminating nucleic acids in a protein sample will increase the apparent absorbance, likewise for contaminating proteins in a nucleic acid sample).

Important aspects of quantification of proteins using UV absorbance

- If a protein contains Trp, Tyr or Cys residues it will absorb in the UV. If it does not contain these amino acids, it will not absorb UV light, and we cannot quantify it using this method
- Multiple Trp, Tyr or Cys residues will contribute to the Extinction coefficient for the protein. Thus, we need to know how many of these residues are present in the protein to know the correct extinction coefficient
- Nucleic acids (DNA, RNA) contaminant will also absorb UV light, as will other proteins with Trp, Tyr and Cys residues. Thus, the sample must be PURE to use UV absorption to quantify a protein

Molar extinction coefficients of Trp, Tyr and Cys amino acids:

Amino Acid	**E_{280nm} (M^{-1} cm^{-1})**
Trp	5690
Tyr	1280
Cys	120

Example 5.3.1: Bovine insulin

Bovine insulin contains 4 Tyr residues, 6 Cys residues and 0 Trp residues. We can determine the expected molar extinction coefficient at 280nm, E_{280nm}, by the following calculation:

E_{280nm} = (0)(5690) + (4)(1280) + (6)(120)

E_{280nm} = 5840 M^{-1} cm^{-1}

Thus, a 1.0M solution of pure bovine insulin would give an absorbance of 5,840 at 280nm (obviously, it would have to be diluted considerably to be read accurately).

A useful expression relating the parameters of E, concentration (C) and A are derived from the Beer-Lambert law (assuming 1cm path length):

A/E = C

For example, if a sample of bovine insulin was observed to give an absorbance at 280nm of 0.745 we could calculate the concentration to be:

0.745/5840 M^{-1} cm^{-1} = C

C = 1.28 x 10^{-4}M (note: cm^{-1} drops out with 1cm pathlength)

It should be noted that a deuterium lamp is required for UV spectrophotometry, as well as quartz cuvettes (since glass absorbs UV light)

Colorimetric (chromogenic) methods of protein concentration determination

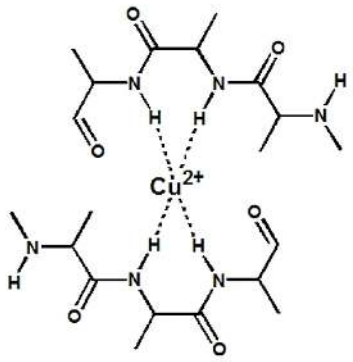
Chromo means color and genesis mean creation, so chromogenic means the "creation of color". Color means color (duh…) and metric means to measure, so colorimetric is to "measure color". Both terms refer to the same sort of thing in the present case - we can modify the protein sample with appropriate reagents so as to produce a color reaction (in visible spectrum) and measure protein concentration using a VIS spectrophotometer. Advantages are:

- Cheap lamp! (tungsten light bulb versus deuterium for UV)
- Cheap cuvette! (cheap glass or plastic versus quartz)
- Not contaminating absorbance from proteins or nucleic acids! (no absorption in VIS spectrum)

We will consider three methods: The **Biuret, Lowry and Bradford** methods of colorimetric determination of proteins.

Biuret

Under high pH (alkaline) conditions the copper II ion (Cu^{2+}) is believed to form a complex with peptide nitrogens of proteins:



Fiugre 5.3.3: *Cu^{2+} complex*

This complex absorbs light at 550nm and has the following useful properties:

- It is dependent upon at least a dipeptide structure (see above), thus, contaminating amino acids will not contribute to the 550nm absorption
- The binding depends upon the peptide backbone nitrogen and not the side chain functional group. Thus, the binding is independent of sequence
- Nucleic acids do not interfere since they don't share the peptide backbone structure
- However, ammonia and certain amines can interfere. Thus, ammonium sulfate salts, and the common biological buffer TRIS (tris hydroxymethyl amino ethane) will provide a false positive and cannot be present in the sample
- Also, the absorbtion is relatively weak, thus, the method is somewhat insensitive and requires a relatively high concentration of protein

Lowry

The Lowry method is a modification of the Biuret method. After treatment with Copper II, the protein is treated with phosphomolybdotungstate mixed acids (acidic compounds of molybdenum and tungsten ions). These acids are known as the **Folin-Ciocalteu (or just Folin) reagent**. The Folin reagent is added under alkaline conditions, and the Folin reagent is subsequently reduced by the the Copper ions as well as Tyr, Trp and polar amino acid side chains. The product of this reaction is **heteropolymolybdenum blue**, which absorbs strongly at 750nm. This assay has the following properties:

- More sensitive than the Biuret assay (can detect lower concentrations of protein)
- Somewhat dependent upon amino acid composition (i.e. relative concentrations of Tyr, Trp and polar amino acids)
- Absobtion reaction is linearly dependent upon protein concentration, but only at low concentrations of protein (i.e. the standard curve and assay must be performed at a low concentration regime).
- Sensitive to contaminants as with the Biuret method, as well as others related to the Folin reagent and redox reactions.
- More critical to timing and precision of person doing the assay

Bradford

A dye known as Coomassie Brilliant Blue was developed by the textile industry. It was noticed to stain skin as well as the textiles. Thus, this dye (which normally absorbs at 465nm) was known to bind to proteins and to absorb strongly at 595nm. The dye forms a wide variety of strong, but non-covalent, interactions including hydrogen bonding donor and acceptor interactions as well as hydrophobic (non-polar) interactions.

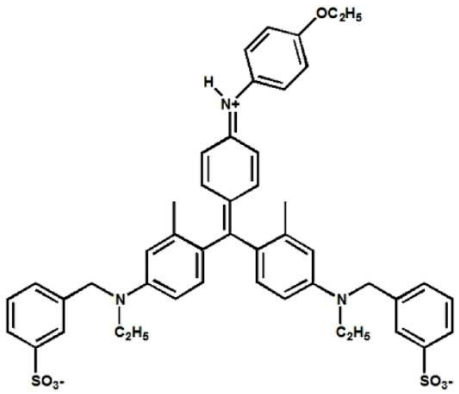


Figure 5.3.4: *Coomassie Brilliant Blue binding*

The method is quite simple: a single step in which the dye is added to the protein solution under acidic conditions, and then the absorbance is read at 595nm. However, the method has the following properties:

- The response is generally independent of the amino acid composition
- The assay is sensitive, but somewhat non-linear. Thus, a standard curve must always be performed (using known concentrations of pure protein)
- The dye stains pretty much everything, including cuvettes, floors, countertops. It can be a real mess if spilled (I know this from personal experience). And since it is a textile dye, if you get it on your clothes, you will need to learn to like blue polka dots.

This page titled 10.4.3: Quantification of Protein Concentration is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **5.3: Quantification of Protein Concentration** by Michael Blaber has no license indicated.

10.4.4: Chromatography

"Chroma" refers to color and "graphy" refers to writing.

Chromatography involves the **physical separation of a mixture of compounds**, where historically the identification of the individual compounds is by their unique color. Chromatography can be used as a purification method (preparative scale) and also for the identification of compounds based on their chromatographic behavior (analytical scale).

There are many types of chromatography, but all involve dissolving the compounds to be separated in a **liquid solution** (the "mobile" phase) and then passing this solution across (or through) some solid support (or "matrix") known as the "stationary" phase. The stationary phase is typically a slurry of solid beads of silica or polysaccharide compounds, or a solid surface of these compounds. As the mobile phase travels through or across the solid phase the dissolved solutes interact to varying degrees with the stationary phase (i.e non-covalent **attractive** forces). **The stronger the attraction, the more the molecules are retarded** in comparison to the mobile phase. If there is no attraction for the solid support, then the solute molecules move with the mobile phase. The different rates of movement for the different solutes results in their separation from each other.

An example you can do at home involves a coffee filter (stationary phase) and water (mobile phase) in the separation and analysis of the dye in a water soluble marker pen (the mixture of compounds). If you draw a line with a purple marker on a strip of the filter paper, and place the bottom in a dish of water, the water will wick up the paper, and the differential affinity of the dye inks for the solid support (cellulose) results in differential migration. In the example below, the red ink has some affinity for the paper, but the blue ink does not (and migrates with the front edge of the mobile phase).



Figure 5.4.1: *Chromatography of purple pen*

Chromatography in biochemistry typically utilizes not paper, but beads of polysaccharide (often chemically derivatized) packed into a column, as the solid support. The solid support is often called the chromatography "resin". Some common types of chromatographic resins include:

- Ion exchange
- Affinity
- Hydrophobic
- Gel filtration

Often, the solutes to be separated are proteins, and so the discussion will focus upon protein chromatography (but the principles are the same for any solute)

Ion exchange

Ion exchange resins contain charged groups.

- These may be acidic in nature (in which case the resin is a cation exchanger)
- or basic (in which case it is an anion exchanger).

- Cation and anion exchangers may be broken down further into weak and strong exchangers (reflecting binding affinity).

Type of exchanger	Functional group	Common name
Weak cation exchanger	carboxymethyl (-)	CM cellulose/sephadex
Strong cation exchanger	sulfopropyl (-)	SP sephadex
Weak anion exchanger	diethylaminoethyl (+)	DE cellulose/sephadex
Strong anion exchanger	quaternary amine (+)	QAE sephadex

Usually, samples are **loaded under low ionic strength** conditions (which promotes electrostatic interactions) and bound material is eluted using either a step or gradient elution of buffer with higher ionic strength.

- Generally speaking, a protein will **bind to a cation exchange resin if the buffer pH is lower than the isoelectric point (pI) of the protein**, **and will bind to an anion exchange resin if the pH is higher than the pI.**
- Knowledge of the pI of the protein is therefore helpful in designing a purification protocol using ion exchange resins (however, you can always simply try different resins to see which works best).

Elution of proteins from ion exchange resins

Proteins bound to ion exchange resins are bound via non-covalent ionic (salt-bridge) interactions. We can compete for these ionic binding sites on the resin with other ionic groups, namely, salts

- There are two general types of methods when eluting with a salt solution: **1. Gradient elution and 2. Step elution**
- A gradient elution refers to a smooth transition of salt concentration (from low to high) in the elution buffer. Weakly binding proteins elute first, and stronger binding proteins elute last (i.e. they require higher salt concentrations in the buffer to compete them off the column)
- A gradient salt concentration can be made using a gradient maker. In its simplest form, this consists of two containers (must be the same shape) connected by a siphon (or tube at the bottom). One container contains the low salt buffer, and the other contains high salt buffer. The buffer is withdrawn from the low salt container:

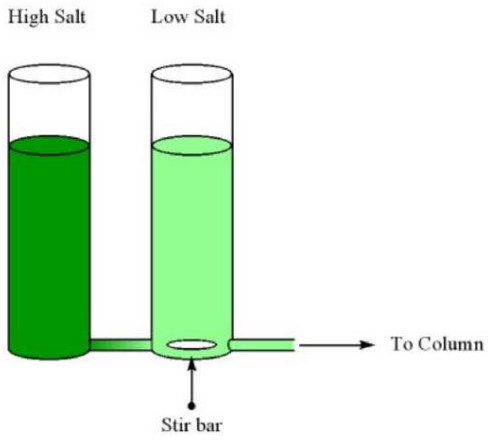


Figure 5.4.2: *Gradient maker*

- This will produce a linear gradient from low to high salt concentrations over the total volume of the gradient
- If we know the concentration range of salt over which a protein of interest will elute we can simply elute with a buffer containing that concentration of salt. This is known as a step elution.
- Step elutions are generally faster to run, and elute the protein in a smaller overall volume than with gradient elutions. They generally work best when contaminants elute at a significantly different salt concentration than the protein of interest

Note that after ion exchange chromatography the protein of interest will be in a buffer with a potentially high salt concentration. This must be taken into account before proceeding with the next step in the purification scheme

Affinity chromatography

Affinity chromatography is a general term which applies to a wide range of chromatographic media. It can be basically thought of as some inert resin to which has been attached some compound which has a *specific affinity* for your protein of interest.

- Thus, a specific antibody attached to an inert resin would be a type of affinity chromatography.
- Other examples might include: a protease inhibitor attached to some matrix, designed to bind a specific protease
- a cofactor bound to some matrix, designed to bind to a particular enzyme
- a metal ion bound to a matrix, designed to chelate a protein with a metal binding site, and so on.

In each case, the type of resins used and the method of attachment may vary, as will the method of elution. One generalization regarding method of elution is that the **bound ligand can be competed off of the column's functional group by including in the elution buffer a high concentration of the free functional group**. For example, if the functional group of the column is a cofactor, then the bound protein can be competed off the column by passing a buffer containing a high concentration of cofactor (or cofactor analog) through the column.

Other methods of elution include changing the buffer conditions such that the protein is no longer in the native state (since it is the native state which confers the structure required for the specific binding interaction). This can be achieved by changing pH or by adding denaturing agents such as urea or guanidine.

With affinity chromatography, typically the purification achieved in a single step can be **dramatic** - on the order of several thousand fold. Single step purifications with specific affinity columns are not unheard - in fact it is an ideal goal of purification - *a matrix which recognizes only the protein of interest and none other*.

Hydrophobic resins

Hydrophobic resins contain a non-polar functional group, such as an alkane or aromatic group.

- Many proteins are able to sequester such groups on their surface and this exclusion from solvent provides the basis of the binding energy (i.e. the "hydrophobic effect").
- This interaction is enhanced by ***increasing ionic strength***, such that proteins may ***bind under high salt conditions*** **and** ***elute under low salt conditions*****.**
- As such these columns may be used to not only provide purification, but to *desalt* samples (for example after an initial ammonium sulfate precipitation).
- It is usually not possible to predict in advance which particular resin will bind a given protein, this is usually determined empirically. However, the longer the alkane, or the larger the aromatic compound, the stronger the binding typically will be.

Due to the nature of hydrophobic interactions and ionic strength, hydrophobic chromatography and ion exchange chromatography can be conveniently used sequentially. For example, after ion exchange the protein is in high salt conditions, thus it can be loaded directly onto a hydrophobic column. Conversely, a hydrophobic column is eluted in low salt, which is a requirement for binding to an ion exchange resin.

A distinction should be noted between hydrophobic interaction chromatography and reverse phase chromatography

- Hydrophobic interaction chromatography is performed in aqueous solvent conditions and changes in ionic strength are used to elute the column. The protein typically binds in the native state via hydrophobic groups located on the surface of the protein. The native state is retained during the elution conditions
- Reverse phase chromatography utilizes a hydrophobic solvent (typically acetonitrile) and the binding of a ligand is a function of the phase partition between the hydrophobic nature of the solvent and column functional group. Proteins are typically denatured in such solvents and bind due to the hydrophobic nature of the entire polypeptide sequence. Since the majority of hydrophobic groups are located in the core of globular proteins, the binding is related to the denaturation of the protein and the accessibility of these groups to the column functional groups. Proteins can be purified using reverse phase chromatography, but usually must be refolded in some way to regain functionality (i.e. the native state)

Gel filtration

Gel filtration does not rely on any chemical interaction with the protein, rather it is based on a **physical** property of the protein - that being the *effective molecular radius* (which relates to mass for most typical globular proteins).

- Gel filtration resin can be thought of as beads which contain pores of a defined size range.
- Large proteins which cannot enter these pores pass around the *outside* of the beads. Therefore, **the volume of the column appears smaller to a large molecule**.
- Smaller proteins which can enter the pores of the beads have a larger volume that they can explore, **thus the volume of the column appear larger to a small molecule**.
- Both large and small molecules experience **the same flow rate of mobile phase** (i.e. L/min).Thus, a sample of proteins passing through a gel filtration column will *separate based on molecular size*: **the big ones will elute first and the smallest ones will elute last (and "middle" sized proteins will elute in the middle)**.

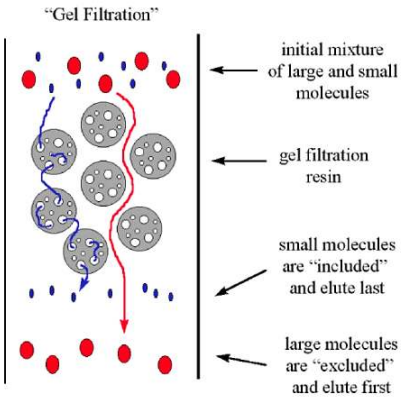


Figure 5.4.3: *Gel filtration*

- If your protein is unusually "small" or "large"*in comparison to contaminating proteins* then gel filtration may work quite well.

Where will a protein elute in a gel filtration experiment?

- There are two extremes in the separation profile of a gel filtration column.
- There is a critical molecular mass (large mass) which will be ***completely excluded*** from the gel filtration beads. All solutes in the sample which are equal to, or larger, than this critical size will behave identically: they will all eluted in the excluded volume of the column
- There is a critical molecular mass (small mass) which will be ***completely included*** within the pores of the gel filtration beads. All solutes in the sample which are equal to, or smaller, than this critical size will behave identically: they will all eluted in the included volume of the column
- Solutes between these two ranges of molecular mass will elute between the excluded and included volumes

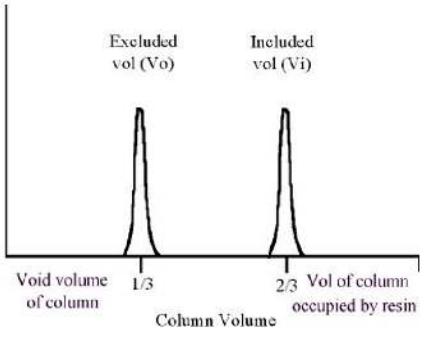


Figure 5.4.4: *Protein elution in gel filtration*

As a general rule of thumb, the excluded volume (Vo) is approximately equal to one third of the column volume, the included volume is approximately equal to two thirds of the column volume (the "missing" third is taken up by the volume of the resin material).

- In gel filtration **the resolution is a function of column length** (the longer the better)
- However, one drawback is related to the maximum sample volume which can be loaded. The larger the volume of sample loaded, the more the overlap between separated peaks. Generally speaking, the sample size one can load is limited to about ***3-5% of the total column volume*.**
- Thus, gel filtration is best saved for *the end stages of a purification* ,when the sample can be readily concentrated to a small volume.
- Gel filtration can also be used to remove salts from the sample, due to its ability to separate "small" from "large" components.
- Finally, gel filtration can be among the most "gentle" purification methods due to the lack of chemical interaction with the resin.

This page titled 10.4.4: Chromatography is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **5.4: Chromatography** by Michael Blaber has no license indicated.

10.4.5: Gel Electrophoresis of Proteins

Gel electrophoresis is used to characterize one of the most basic properties - **molecular mass** - of both polynucleotides and polypeptides. Here we will focus exclusively on gel electrophoresis of proteins

Gel electrophoresis can be used to determine:

- the *purity*of a protein sample
- *heterogeneity* and extent of degradation of a protein sample
- *subunit composition* of a protein sample

How does it work?

The underlying principle of electrophoresis is the **migration property of charged species within an electric field**. Thus, it is the simple behavior of opposite charges attracting. An electric field is established across the electrodes of a power supply, and charged ions move in this electric field. Note that in such an apparatus, the word "ANODE" refers to the positively charged electrode (the ANODE attracts ANIONS) and the word "CATHODE" refers to the negatively charged electrode (the CATHODE attracts CATIONS). The cathode and anode terms are consistent with their redox reaction definitions in that reduction is subsequently occurring at the cathode and oxidation is occurring at the anode:

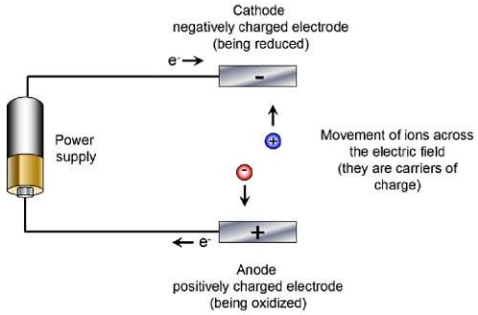


Figure 5.5.1: *Anode and cathode*

Warning: read the following note only if you are curious about batteries and labeling of electrodes, otherwise, don't bother (it may only be confusing).

> **Note**
>
> Redox chemistry within the external power supply is driving the redox reaction at the electrodes. Note that the electrons from the anode are going to the "+" terminal of the battery. Reduction at this electrode of the battery must be occurring and is driving the oxidation at the anode of the external electrodes. If this terminal of the battery is being reduced, then it must be the cathode in the redox reaction within the battery. Thus, batteries have their cathode labeled as "+" and anode labeled as "-". Took me hours to figure this out.

Proteins are comprised of the 20 common amino acids, which include both negatively charged (i.e. acidic) side chains (e.g. aspartic acid, glutamic acid) and positively charged (i.e. basic) side chains (e.g. histidine, lysine and arginine). Thus, proteins can be charged, and will migrate in an electric field.

At this point there are a couple of things to consider:

1) Any such separation is a non-equilibrium process. By this, we mean that if we let the process continue on until some equilibrium condition is met, all the anions will be on one electrode and all the cations will be on the other. It would be better to halt the separation process at some intermediate time point to permit achieve separation:

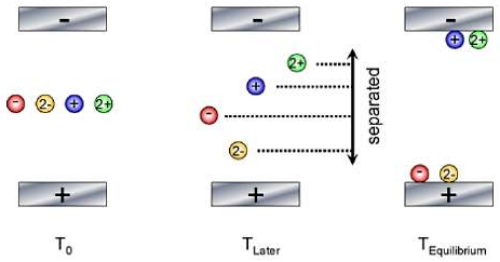


Figure 5.5.2: *Separation process*

2) The other problem is that once the electric field is switched off, diffusion will cause the separated ions to move around (i.e. we will lose the separation we have tried to achieve). To solve this problem, the separation is not performed in solution, but within a matrix (i.e. a molecular mesh or network). The matrix provides a **frictional component** that resists diffusion. Furthermore, the friction of the matrix is an important factor in the rate of migration of the ions.

3) Note also that separation is achieved by initial application of the sample within a narrow zone (band). If the sample is initially dispersed, although the ions will move, they won't be neatly separated.

Factors that influence the rate of electrophoresis migration (R_f)

Three factors affect the rate of migration:

· Strength of the electric field, E (directly proportional to migration rate)

· Charge on the ionic species, q (directly proportional to migration rate)

· Frictional coefficient of the support matrix, f (inversely proportional to migration rate)

$R_f \alpha qE/f$

These factors can be varied in the following way:

· The strength of the field is a function of the **voltage** of the power supply. Thus, we can vary the voltage directly. In a related issue, the voltage is proportional to the resistance across the electrodes. Current comes into play here also, but in short, it is difficult to achieve high voltage across the electrodes if the resistance is low. The resistance of a solution is **inversely proportional to the ionic strength** (i.e. concentration of ions). Thus, with high salt concentrations, the resistance is low, it is difficult to achieve high voltage, and the migration rate will decrease.

· The charge on an ionic molecule is a function of the **pI of the molecule and the pH of the solution**

If pI > pH the molecule is cationic
(migrates towards cathode)

If pI < pH the molecule is anionic
(migrates towards anode)

If pI = pH the molecule is neutral
(no migration in electric field)

We can therefore alter the migration rate (and possibly the direction of migration) by altering the pH of the solution

· The frictional coefficient of the matrix can potentially be altered. The matrix is typically a polymer network (see below), referred to as a **gel**, and the frictional coefficient can be increased by increasing the polymer concentration

The gel matrix for gel electrophoresis of proteins

Gel electrophoresis of proteins almost exclusively utilizes **polyacrylamide**. This is a polymer comprised of two covalently-linked components:

- acrylamide
- bis acrylamide.

The bis acrylamide is essentially a **cross-linking** component of the acrylamide polymer. A typical value for the acrylamide:bisratio is 19:1 and the ***total*** acrylamide concentration in the gel affects the migration of proteins through the matrix (i.e. determines the frictional coefficient).

- **High molecular mass proteins** are separated using low frictional coefficient (i.e. **low concentrations**) of polyacrylamide.
- **Low molecular mass proteins** are separated using high frictional coefficient (i.e. **high concentrations**) of polyacrylamide.
- Although we now have everything in place to perform a gel electrophoresis experiment to separate proteins, there is another consideration that often is undesireable (although sometimes useful). This situation is the fact that different proteins have different charges for a given value of pH. Thus, proteins will migrate as a function of **both** their **mass** (big ones move slowly) and their **overall net charge**.
- If there were some way to cause each protein to have an **identical** charge to mass ratio, we could separate a mixture of proteins **based only upon mass effects**

The role of sodium dodecyl sulfate detergent in polyacrylamide gel electrophoresis

Sodium dodecyl sulfate (**SDS**; also known as "laurel sulfate") is an ionic detergent with the following structure:

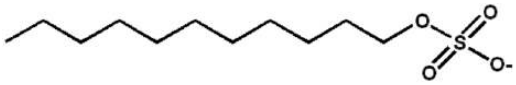


Figure 5.5.3: *Sodium laurel sulfate structure*

- The SDS binds, via hydrophobic interactions, to the proteins in a stoichiometry ***approximately proportional to the size of the protein*** (i.e. a small protein will bind a few molecules, and a large protein will bind a lot of molecules of SDS)
- Due to the charged nature of the SDS molecule the proteins thus will have an approximate ***constant charge to mass ratio***due to the charge provided by the SDS, and will migrate through the gel at a rate proportional to their molecular mass
- The proteins migrate towards the ***anode*** since the charge on the SDS is negative under all pH conditions except highly acidic.

Protein gels are usually performed under ***denaturing conditions,*** meaning that the sample preparation involves heating the protein in the presence of SDS to fully unfold the protein and permit binding of SDS throughout the length of the polypeptide. Once SDS has been bound, the characteristic pI values of the proteins is no longer relevant; the protein takes on a negative charge, and each protein has essentially the same charge to mass ratio.

Migration rate, protein mass, and the % acrylamide in the gel

The greater the percent acrylamide in the gel support, the greater the frictional coefficient, and the slower the migration rate. If the proteins to be separated are of a high molecular mass, and if the % gel is high, the proteins may not even enter the gel (due to overwhelming friction). Thus, it **is essential to match the % gel to the mass of the proteins being separated**. The following table provides a general guideline:

Acrylamide	Range of separation of Polypeptides (length in amino acids)
8%	**25-200 kDa (225-1800 a.a.)**
10%	**15-100 kDa (135-900 a.a.)**
12.5%	**10-70 kDa (90-630 a.a.)**
15%	**6-60 kDa (55-550 a.a.)**
20%	**4-40 kDa (36-360)**

In setting up the SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) experiment we **need to know when to stop the experiment** (since it is not an equilibrium process). This is somewhat difficult to determine since proteins (even with SDS bound) do not absorb in the visible spectrum (i.e. we cannot simply look at the gel to determine when the proteins have been separated). Therefore, it is common to include in the protein sample a **small anionic dye molecule (e.g. bromophenol blue)**:

- The size of the dye molecule is chosen to be very small so that there is essentially **no friction** coefficient with the gel
- The dye is chosen to be **anionic** so that it will migrate in the same direction as the protein/SDS complexes (i.e. towards the anode)
- Since the dye is anionic, and small, it will **migrate the fastest** of any component in the mixture to be separated
- The dye molecule is also chosen so as to absorb in the **visible** spectrum (and, hence, be visually detectable while the gel is running)
- The protein/SDS/dye mixture is loaded on the top of the gel (i.e. cathode side) and when the dye molecule (the "dye front") **reaches the bottom of the gel, the power is turned off and the experiment halted**

Visualization of the separated proteins

Although the gel support provides some friction to molecular motions, as soon as the power is turned off the separated protein bands will begin to diffuse (they are freely soluble in aqueous solution). To prevent this, the gel is treated with an acetic acid and methanol solution which causes almost all proteins to precipitate (become insoluble). This is called "fixing" the gel. Now the separated proteins will not diffuse.

The fixed proteins are, however, still invisible and must be visualized by staining. A common protein stain is Coomassie Brilliant Blue R-250 (related to the dye used previously in the Bradford assay). The fixed gel is incubated in a solution of "Coomassiestain" and then the stain is washed out of the gel by incubation in a weak solution of acetic acid and methanol. The stain will not bind to the acrylamide, and will wash out (leaving a clear gel). However, it remains strongly bound to the proteins in the gel, and these take on a deep blue color.

Determination of molecular mass

With SDS treatment, the proteins will migrate as a function of their molecular mass. The approximate molecular mass of the separated proteins is therefore a function of their **migration distance**. If a series of proteins with different and known molecular masses is analyzed on an identical gel, under identical conditions, then a standard curve can be established that can be used to determine the molecular mass of an unknown protein. A typical "molecular weight standard" includes the following mixture of proteins:

Protein	Molecular Mass (kDa)
Phosphorylase B	**94**
Bovine Serum Albumin	**67**
Ovalbumin	**43**
Carbonic Anhydrase	**30**
Soybean Trypsin Inhibitor	**20.1**
a-Lactalbumin	**14.4**

The molecular mass is quantified as follows:

- Measure (in cm) the migration distance of the **dye front**
- Measure (in cm) the migration distance of the **protein** (will always be less than the dye front)
- Divide the protein migration distance by the dye front distant to get the **relative mobility** value (always < 1)
- Plot the relative mobility value (along x-axis) versus the **log** of the molecular mass (along y-axis)
- The relationship between the relative mobility and the log of the molecular mass should be a linear function - thus, providing a standard curve against which the molecular mass of an unknown protein can be determined from its relative mobility

Here is an example of a molecular mass standard, and two unknown samples, analyzed on a 10% SDS PAGE stained with Coomassie:

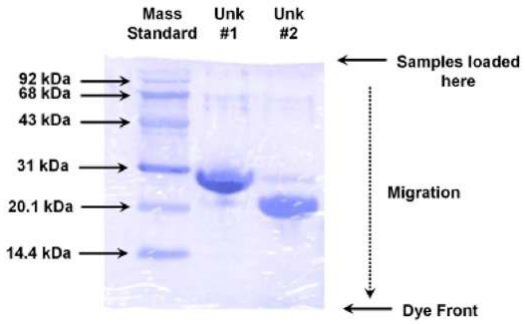


Figure 5.5.4: *Example gel electrophoresis*

The calculation of migration distances, relative mobility and relationship to Log of the molecular mass yields the following:

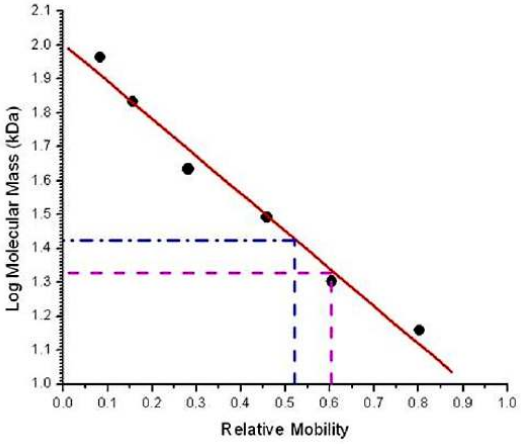


Figure 5.5.5: *Relative mobility and molecular mass*

- Unknown #1 has a value of 1.43 for the log of the molecular mass, or a mass of ~26.9 kDa
- Unknown #2 has a value of 1.33 for the log of the molecular mass, or a mass of ~21.4 kDa
- A visual check of the unknowns versus the standards in the above gel indicate the calculations appear to be correct

There are other variations of SDS PAGE including:

- Discontinuous gels (i.e. a composite of one gel on top of another) to achieve "focusing" of protein bands (i.e. sharper bands, yielding greater sensitivity and resolution)
- Gradient gels (instead of pouring a single % polyacrylamide gel, pour a gradient with a high concentration at the bottom, and a lower concentration towards the top. This permits resolution of a wider range of molecular mass samples)
- Silver staining (a more sensitive type of stain in comparison to Coomassie - allowing detection of lower concentrations of proteins)
- Transfer of resolved protein bands to a secondary support (e.g. nitrocellulose) for probing with other reagents (i.e. antibodies)
- PAGE (no SDS) of native proteins (to allow detection of non-covalent complexes of two or more proteins - since SDS will disrupt such complexes)
- SDS PAGE with the addition of reducing agents (e.g. b-mercaptoethanol, dithiothreitol, etc.). These reagents will reduce disulfide bonds and separate polypeptide chains that are connected by such bonds

However, the above information covers the basics.

This page titled 10.4.5: Gel Electrophoresis of Proteins is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **5.5: Gel Electrophoresis of Proteins** by Michael Blaber has no license indicated.

10.4.6: Introduction to Protein Purification

A successful protein purification procedure can be nothing short of amazing. Whether you are starting off with a recombinant protein which is produced in *E. coli*, or trying to isolate a protein from some animal or plant tissue, you are typically starting with a complex mixture of proteins, nucleic acids, polysaccharides, lipids, etc. from which you may have to extract milligram (or microgram!) quantities of the desired protein, often with high purity, and hopefully with high yield.

Protein purification can be thought of as a series of **fractionation steps** designed so that:

- The protein of interest is found almost exclusively in one fraction (and with good yield)
- A significant amount of the contaminants can be found in a different fraction

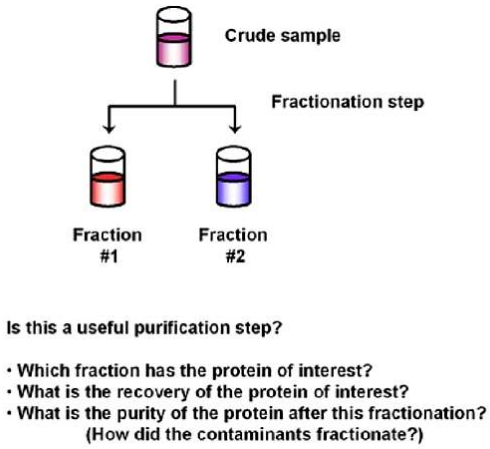


Figure 5.6.1: *Fractionation*

The first step in any purification is the development of an assay for the protein of interest

The assay can be based upon some **unique characteristic** of the protein of interest, possibly involving:

- Enzymatic activity
- Immunological activity
- Physical characteristics (e.g. molecular mass, spectroscopic properties, etc.)
- Biological activity
- Ideally, an assay should be
  - ***specific*** (you don't want a false positive or false negative)
  - ***rapid*** (you don't want to wait a week for the results)
  - ***sensitive*** (you don't want to consume all your sample in order to assay it)
  - ***quantitative*** (you need an accurate way to measure the quantity of your protein at each step in the purification)

During purification you will **need to keep track of several parameters**, including:

1. *The total volume of your sample*
2. *The total amount of protein in your sample*

· can be estimated by using $E^{1\%}_{280nm}$ =14.5

· In other words, measure absorbance at 280nm, divide by 1.4, and you will have the approximate protein concentration in mg/ml

3. *The total amount of the protein of interest (the one you are trying to purify). This information will be determined from your quantitative assay*

This basic information will allow you to keep track of the following parameters during each step of purification:

1. *% yield* for each purification step
2. *Specific activity* of the desired protein ("units of activity" of desired protein/mg total protein)

3. *Purification enhancement* of each step (e.g. "3.5x purification)

In designing a purification scheme you typically have to balance *purification* with *yield*.

- For example, it may be relatively straightforward to obtain 90% pure material with good yield.
- However, it may be difficult to improve that purity by an additional few percentile and still maintain a good yield.
- ***The planned application of the purified protein determines the target purity***.
- If the protein is to be used to determine amino acid sequence information, maybe 90% is acceptable. However, if the material is to be used in clinical trials, 99.999+% may be the target purity.

Initial steps in purification

- Typically (but not always) the protein of interest is contained within the cytoplasm of a cell (sometimes, however, it might be secreted by the cell into the extracellular environment). Thus, cells must be broken open to release the cytoplasm, a process known as cell lysis.
- It is extremely helpful to have some information not only on the general physical and chemical characteristics of the protein you are trying to purify, but also on the *contaminating* components.
- For example, many *E. coli* proteins are generally *low molecular weight* (<50,000 Da) and somewhat *acidic* in isoelectric point

A typical general outline of a protein purification procedure might look like this:

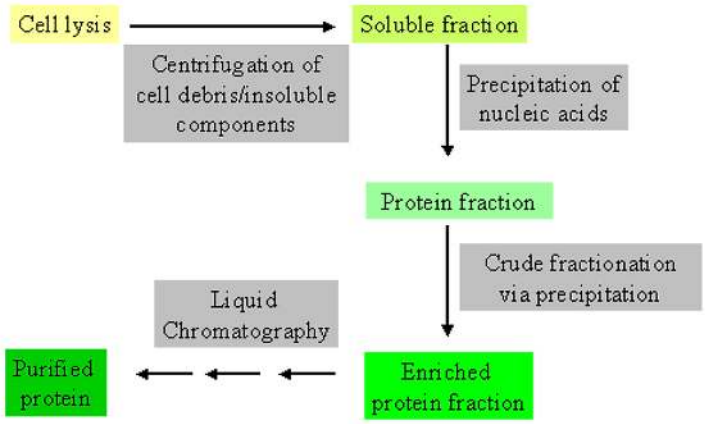


Figure 5.6.2: *Protein purification steps*

Usually the initial steps in purification make use of general physical and/or chemical differences between soluble proteins and other cell components.

- For example, soluble proteins can be separated from general cellular debris, and intact cells, by *centrifugation*.
- Thus, cells are physically disrupted (via homogenization or a cell press) to allow release of cell contents. This is then followed by centrifugation to separate generally soluble components from those which are insoluble.
- It is at this point that data collection begins in order to monitor the purification.

Nucleic acids can sometimes be readily removed from the sample by the addition of large cationic compounds such as *polyethylene imine*, or *streptomycin sulfate*.

- The nucleic acids bind to these compounds via electrostatic interactions and the complex precipitates and can be removed via centrifugation.
- The same general result can be obtained by mixing in ion exchange resins which are *anion exchangers* (i.e. the resins contain cationic groups) and then filtering or centrifuging to remove. As with either method, it should be confirmed that the desired protein is *not* bound as well.

Crude fractionations of proteins can be achieved by adding various quantities of precipitants such as *ammonium sulfate*, or*polyethylene glycol* (PEG).

- For this type of purification step an initial experiment is performed to monitor the fraction of overall protein, as well as desired protein, remaining in solution (and pellet) as a function of precipitant concentration.

Ammonium Sulfate (% saturated)	**0**	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**
Sample A_{280} (in solution)	10000	9000	6000	2000	1000	75	50	40	25	20
Activity assay(units) (in solution)	2000	2000	2000	1900	1700	100	30	5	0	0

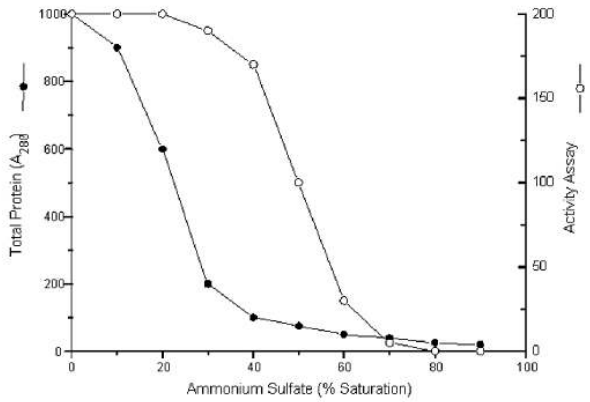


Figure 5.6.3: *Protein concentration and precipitant concentration*

How can we use this information to utilize ammonium sulfate precipitation as a useful purification step in our overall purification procedure?

Let's begin by calculating the **specific activity** (a measure of purity)of the **starting sample:**

Specific activity = total units of desired protein / mg of total protein

To calculate these parameters we need to know the total sample volume (in addition to the information in the above table). In this case, the total sample volume is 1 L. Knowing this, and the absorbance, we can determine the total protein in the sample:

Sample A_{280}/1.4 » mg/ml of sample

1000/1.4 = 714 mg/ml

714 mg/ml * 1.0L(1000ml/L) = **714,000 mg total protein**

The protein of interest was assayed at 200 units present in the starting sample. Therefore, the specific activity of the starting sample is:

Specific activity = 200 units / 714,000 mg = **2.80×10^{-4} units/mg**

Note

Note: the "units" of activity depend upon the protein of interest. It could be a measure of enzymatic activity - i.e. 1.0 unit of enzymatic activity results in 1mmol of substrate being consumed in 1 min time.

We can now expand upon the above table to include the specific activity of the sample

Ammonium Sulfate (% saturated)	**0**	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**
Sample A_{280} (in solution)	1000	900	600	200	100	75	50	40	25	20
Total mg protein (in solution)	714,000	643,000	429,000	143,000	71,400	53,600	35,700	28,600	17,900	14,300
Activity assay(units) (in solution)	200	200	200	190	170	100	30	5	0	0
Specific activity (units/mg)	$2.80x10^{-4}$	$3.11x10^{-4}$	$4.66x10^{-4}$	$13.3x10^{-4}$	$23.9x10^{-4}$	$18.7x10^{-4}$	$8.40x10^{-4}$	$1.75x10^{-4}$	0	0

We can plot the specific activity of the sample remaining in solution for the different concentrations of added ammonium sulfate:

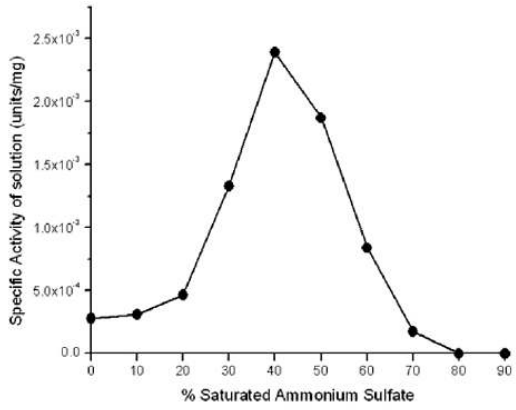


Figure 5.6.4: *Specific activity and precipitant concentration*

- The highest specific activity is observed in solution when ammonium sulfate is added to 40% saturation
- What is the increase in purity for the 40% solution sample?

Purification = final specific activity / initial specific activity

Purification = ($23.9x10^{-4}$ units/mg) / ($2.80x10^{-4}$ units/mg) = 8.5-fold purification

However, purification is not the entire story. We also need to consider yield:

Yield (%) = (final total units / initial total units) x 100

And we can add this information our purification statistics:

Ammonium Sulfate (% saturated)	**0**	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**
Sample A_{280} (in solution)	1000	900	600	200	100	75	50	40	25	20
Total mg protein (in solution)	714,000	643,000	429,000	143,000	71,400	53,600	35,700	28,600	17,900	14,300
Activity assay(units) (in solution)	200	200	200	190	170	100	30	5	0	0
Specific activity (units/mg)	2.80×10^{-4}	3.11×10^{-4}	4.66×10^{-4}	13.3×10^{-4}	23.9×10^{-4}	18.7×10^{-4}	8.40×10^{-4}	1.75×10^{-4}	0	0
Purification	N/A	1.1	1.7	4.8	8.5	6.7	3.0	0.6	-	-
Yield (%)	100	100	100	95	85	50	15	2.5	0	0

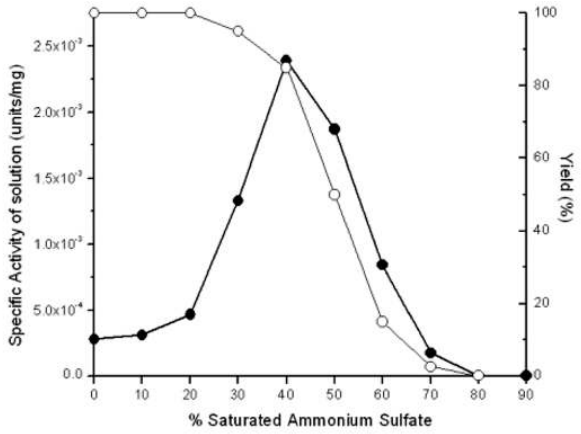


Figure 5.6.5: *Percent yield and precipitant concentration*

Information on the yield shows that our protein of interest begins to precipitate around 30% ammonium sulfate. Thus, while the addition of ammonium sulfate to 40% provides the greatest purification, it is associated with a 15% loss of our protein of interest. Another option is to added ammonium sulfate to 30%. In this case, we would realize a purification of 4.8-fold, and suffer losses of only 5%.

At each step of a purification scheme we are typically faced with a decision regarding a tradeoff between purity and yield

- If the starting material is cheap, plentiful, or easy to obtain, **we may choose our fractionation steps so as to maximize purity at the expense of yield**
- If the starting material is expensive, limited, or difficult to get, **we may choose to maximize yield at the expense of purity**
- Again, these decisions are also determined by the intended purpose of the final material, and therefore, the final purity desired.

Thus, in the above experiment, we would choose to add ammonium sulfate to a concentration between 20-30% if we wanted to maximize yield, or perhaps 40% (or slightly higher) if we wanted to maximize the purification.

Practical matters with ammonium sulfate fractionation steps

We would probably like to remove the ammonium sulfate from our sample (perhaps the next purification procedure cannot tolerate high ionic strength buffers). Notice that from the data in the above table it looks like all our protein of interest is precipitated if ammonium sulfate is added to a concentration of 80%. Thus, this we can use this precipitation property to remove our protein from most of the ammonium sulfate:

1. Add ammonium sulfate to our sample to a concentration of 20-40% saturation (the exact percentage being determined by whether we wish to maximize purity or yield)
2. Centrifuge and **discard the pellet**
3. Add ammonium sulfate to 80% saturation to the retained solution
4. Centrifuge and **keep the pellet**. Resuspend the pellet in buffer to solubilize the protein.

This page titled 10.4.6: Introduction to Protein Purification is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **5.6: Introduction to Protein Purification** by Michael Blaber has no license indicated.

SECTION OVERVIEW

10.5: 6. Lab Notes Part 2

10.5.1: Genetic Transformation (using bacteria and the pGLO plasmid)

10.5.2: Enzyme kinetics

10.5.3: Ligand binding

10.5.4: Restriction Mapping

10.5.5: Polymerase Chain Reaction (PCR)

10.5.6: Use of PC and internet for biochemical research

This page titled 10.5: 6. Lab Notes Part 2 is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

10.5.1: Genetic Transformation (using bacteria and the pGLO plasmid)

Genetic transformation is the process by which an organism acquires and expresses a new gene. Genetic engineering is the directed transfer of a gene, or piece of DNA, into a cell (typically a bacteria). Typically the intent is to force the cell to express (produce) the protein that the newly introduced piece of DNA codes for (known as heterologous expression). The organism commonly used for genetic transformation and heterologous expression of human genes/proteins is the single celled bacteria known as ***Escherichia coli*** (E. coli). This organism has several traits of importance in the laboratory:

- Single cell organism
- Doubling time is **20 minutes** (in rich media) to 1 hour (minimal media)
- Naturally lives in human gut (part of normal intestinal flora). Thus, **normal growth temperature is 37°C** (human body temperature)
- *E. coli* has a **single chromosome** that is a **circular DNA** molecule
- *E. coli* is **gram negative** - has a inner cytosolic membrane, periplasmic space, and outer cell membrane

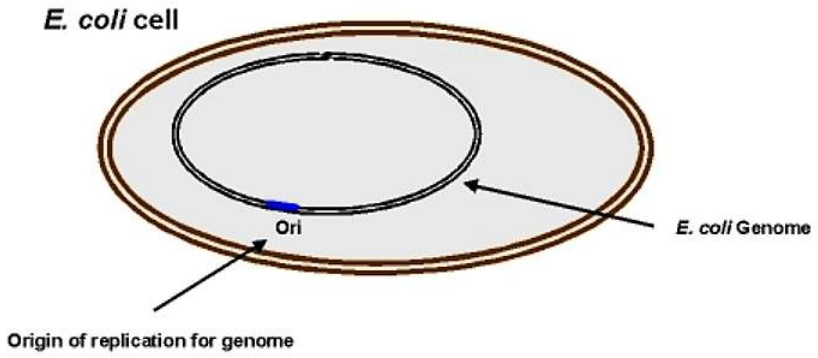


Figure 6.1.1: *E. Coli genome*

Foreign DNA can be introduced into the E. coli as a second "chromosome", or as another circular DNA molecule that can **replicate autonomously** due to having its **own origin of replication**. Such "autonomously replicating elements" are referred to as "**plasmids**" or "**vectors**".

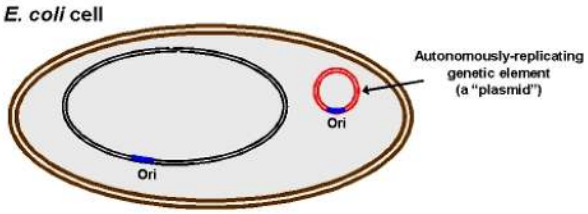


Figure 6.1.2: *Plasmid in E. Coli*

In order to "**stably retain**" the plasmid, there needs to be some type of metabolic reason for the *E. coli* to keep the plasmid around. If the plasmid contains a gene that codes for a protein that protects against antibiotics, then, only cells that have the plasmid will survive in the presence of that antibiotic. Drug resistance can therefore form the basis of a "**selectable marker**" for the presence of the plasmid in a sample of *E. coli.* Ampicillin is an antibiotic for gram negative cells such as E. coli (it is part of the penicilln family of antibiotics):

- Production of the outer cell membrane/proteoglycan structure is inhibited In the presence of ampicillin (a lethal event for the bacteria)
- Ampicillin, as a molecule, contains a **b-lactam ring** structure. This ring can be cleaved (and the ampicillin destroyed) by the enzyme b-lactamase
- b-lactamase enzyme is the product of the ***bla*** gene (genes are typically lower case and italicized)
- If a plasmid contains the ***bla*** gene, it will confer resistance to ampicillin to the host E. coli

- Such *E. coli* grown in the presence of ampicillin will be **selected for.** Under these conditions, any wild type E. coli in the sample will be **selected against**)
- Thus, ampicillin resistance is a **selectable marker** for the **plasmid**

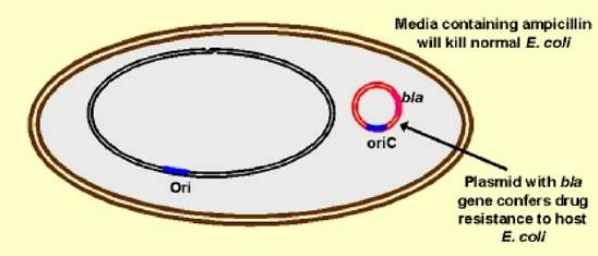


Figure 6.1.3: *Ampicillin resistance plasmid*

Other genes (that express other proteins) can now be introduced into the plasmid, and the host E. coli forced to express the protein of interest

pGLO

Plasmids are typically abbreviated with an acronym that begins with the lower case "p", and the name can provide some information regarding the person that designed the plasmid, or the contents of the plasmid. The pGLO plasmid contains an origin or replication, a selectable marker, and the gene for **Green Fluorescent Protein (GFP)**. The plasmid also contains a gene for the arabinose C protein, which is a protein that regulates expression from the arabinose BAD promoter (P_{BAD}). Promoters are usually indicated with an acronym that begins with an upper case "P".

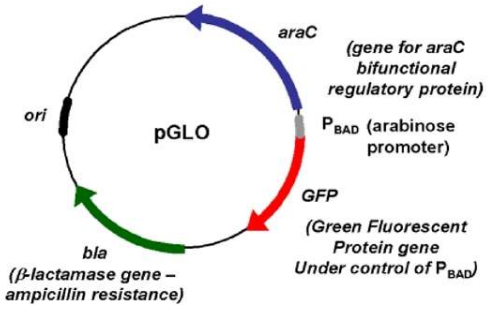


Figure 6.1.4: *pGLO Plasmid. The* ***ori*** *is the origin of repliation for the pGLO plasmid,* ***bla*** *is the gene that codes for b-lactamase, and is the selectable drug-resistant marker for the plasmid,* ***GFP*** *is the GFP gene and* ***araC*** *is the gene coding for the arabinose C protein*

A "**promoter**" is a region of DNA that signals RNA polymerase to initiate **transcription** (for production of mRNA). Promoters are typically located at the **start** (5' end) of a gene that codes for a protein (since mRNA production proceeds 5'->3'). The bla gene includes a promoter at the 5' end of the gene. This is a weak constitutive promoter (always "on" at a low-level). It will instruct RNA polymerase to continually make a low-level of mRNA for this gene. The mRNA will be translated to produce low-levels of the b-lactamase protein. The GFP is transcribed by the **arabinose P_{BAD} promoter**. This is a **strong** promoter and will cause RNA polymerase to make a large number of copies of mRNA from this gene (and therefore, a lot of GFP protein). However, the arabinose P_{BAD}promoter is **regulated** by the protein coded for by the *araC* gene (which has its own promoter, much like the *bla* gene):

- In the **absence** of the sugar **arabinose**, the araC protein binds to the PBAD promoter and **prevents transcription** (of *GFP*)
- In the **presence** of **arabinose** in solution, the *araC* protein binds the arabinose, and this results in a conformational change to the *araC* protein, the result of which is that it now instructs RNA polymerase to make **many copies** of the ***GFP*** mRNA (and thus, a lot of GFP protein)

How is the pGLO plasmid introduced into the E. coli cell?

The general process by which foreign DNA is introduced into a cell is called **transformation**. There are several ways to transform DNA into an E. coli cell, but the most common way is by making the cells **competent**. "Competent" cells have the ability to take up DNA molecules from the environment. Normal *E. coli* are **not** competent, however, if they are treated with a solution of **calcium chloride** their cell membranes become competent. Actually, only a small fraction of the cells treated with $CaCl_2$ are able to take up foreign DNA, however, since the number of cells in a sample is large, the low efficiency of transformation is not much of a problem. The selectable markers ensure that only the cells that did take up the foreign DNA (i.e. plasmid) will survive and grow.

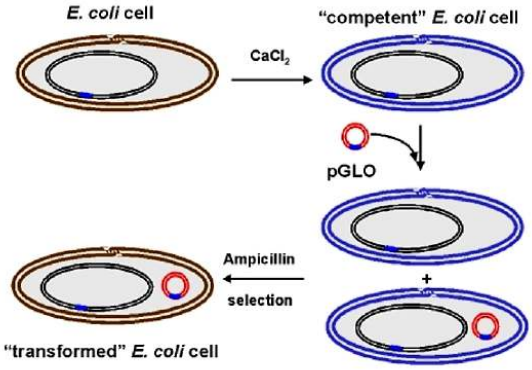


Figure 6.1.5: *Transformation of E. Coli*

Expression of GFP

Addition of arabinose sugar to the growth media will cause RNA polymerase to start transcribing the GFP gene (i.e. making mRNA molecules). The cellular machinery (e.g. ribosomes) will translate this mRNA into corresponding GFP protein.

- GFP will fluoresce green under UV light
- The presence of green light from E. coli cells indicates that the transformation (and drug resistance selection) has been successful
- GFP is a gene from a jelly fish and is the reason that some jelly fish glow green. It is not a normal gene for E. coli, however, if introduced into E. coli, it will make the GFP protein (and fluoresce green)

This page titled 10.5.1: Genetic Transformation (using bacteria and the pGLO plasmid) is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **6.1: Genetic Transformation (using bacteria and the pGLO plasmid)** by Michael Blaber has no license indicated.

10.5.2: Enzyme kinetics

Enzymes are **protein catalysts,** they influence the **kinetics** but **not the thermodynamics** of a reaction

- Increase the rate of a chemical reaction
- Do not alter the equilibrium

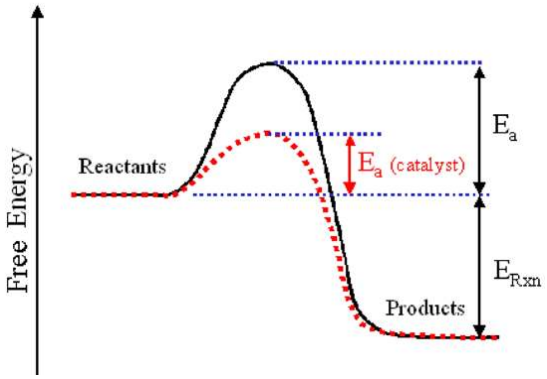
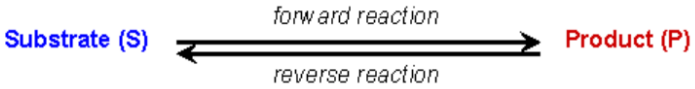


Figure 6.2.1: *Catalyst activity*

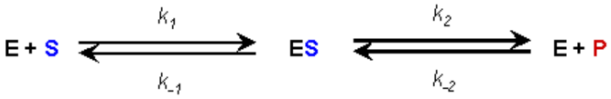
- They increase the rate by stabilizing the transition state (i.e. lowering the energy barrier to forming the transition state (they do not affect the energetics of either the reactant(s) or product(s)

Michaelis-Menten derivation for simple steady-state kinetics

The Michaelis-Menten equation is a **mathematical model** that is **used to analyze simple kinetic data**. The model has certain **assumptions**, and as long as these assumptions are correct, it will accurately model your experimental data. The derivation of the model will highlight these assumptions.

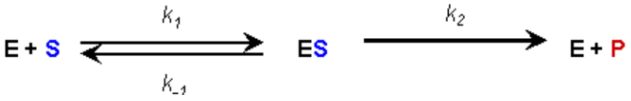


In an enzyme catalyzed reaction the substrate initially forms a reversible complex with the enzyme (i.e. the **enzyme and substrate have to interact** for the enzyme to be able to perform its catalytic function). The standard expression to show this is the following:



ASSUMPTION #1:

- There is no product present at the start of the kinetic analysis
- Therefore, as long as we monitor **initial reaction rates** we can **ignore** the reverse reaction of **E+P going to ES**



ASSUMPTION #2:

- During the reaction an **equilibrium** condition is established for the binding and dissociation of the Enzyme and Substrate (Briggs-Haldane assumption)
- Thus, the **rate of formation** of the ES complex is equal to the **rate of dissociation plus breakdown**

ASSUMPTION #3:

- **[E] << [S]**
- The enzyme is a catalyst, it is not destroyed and can be recycled, thus, only small amounts are required
- The amount of S bound to E at any given moment is **small** compared to the amount of free S
- It follows that **[ES] << [S]** and therefore **[S] is constant** during the course of the analysis (NOTE: this assumption requires that the reaction is monitored for a **short period**, so that not much S is consumed and [S] does not effectively change - see next assumption)

ASSUMPTION #4:

- Only the **initial velocity** of the reaction is measured
- [P] = 0 (reverse E + P reaction can be ignored)
- [S] » $[S]_{initial}$

ASSUMPTION #5:

- The enzyme is either present as free enzyme or as the ES complex
- $\mathbf{[E]_{total} = [E] + [ES]}$

Michaelis-Menten derivation using above assumptions:

Rate of ES formation = k_1[E][S] + k_{-2}[E][P]

Assumption #1 says we can ignore the k_{-2} reaction, therefore:

Rate of ES formation = k_1[E][S]

Assumption #5 says $[E] = [E]_{total} - [ES]$, therefore:

Rate of ES formation = $k_1([E]_{total} - [ES])[S]$

The rate of ES breakdown is a combination of the dissociation and the conversion to product:

Rate of ES breakdown = k_{-1}[ES] + k_2[ES]

Rate of ES breakdown = $(k_{-1} + k_2)$[ES]

Assumption #2 says the rate of ES formation equals the rate of breakdown:

$\mathbf{k_1([E]_{total} - [ES])[S] = (k_{-1} + k_2)[ES]}$

Rearrange to define in terms of rate constants:

$\mathbf{([E]_{total} - [ES])[S] / [ES] = (k_{-1} + k_2) / k_1}$

$\mathbf{([E]_{total}\,[S] / [ES]) - [S] = (k_{-1} + k_2) / k_1}$

Define a new constant, $\mathbf{K_m = (k_{-1} + k_2) / k_1}$

$\mathbf{([E]_{total}\,[S] / [ES]) - [S] = K_m}$

Solve for the [ES] term (for reasons that will be given in the next step):

$\mathbf{[ES] = [E]_{total}\,[S] / (K_m + [S])}$

The actual reaction velocity measured at any given moment is given by:

$\mathbf{V = k_2[ES]}$

Multiple both sides of the above equation by k_2:

$\mathbf{k_2[ES] = k_2[E]_{total}\,[S] / (K_m + [S])}$

thus

$\mathbf{V = k_2[E]_{total}\,[S] / (K_m + [S])}$

The maximum possible velocity (Vmax) occurs when all the enzyme molecules are bound with substrate [ES] = $[E]_{total}$, thus:

$V_{max} = k_2[E]_{total}$

Substituting this into the prior expression gives:

$V = V_{max}[S] / (K_m + [S])$

This is the mathematical expression that is used to model your experimental kinetic data

It is known as the Michaelis-Menten equation

Experimental approach

The general approach is to add a **known concentration of substrate** to the enzyme and to determine the **initial reaction rate** for that concentration of substrate

- Reaction rates are typically given as moles (or micromole) of product produced per unit of time (sec or min) per mole (or micromole) of enzyme
- The experiment is repeated for a wide range of substrate concentrations
- A table of [S] versus V datapoints are collected
- These datapoints are plotted (V versus S) and should fit a curve that agrees with the Michaelis-Menten equation

The V_{max} and K_m terms are **intrinsic** properties of the particular enzyme/substrate combination that you are studying

- They will be determined from the features of the V versus S plot

V_{max}

There are a limited number of enzyme molecules and they can only perform a single reaction at a time. Thus, at **high [S]** the enzymes can be **saturated**

- Under saturating conditions the reaction is going as fast as it can, and additional increases in [S] do not increase the reaction rate.
- The maximum observable rate is V_{max} and the data will **asymptote to this value** at high [S]
- At low [S] the reaction rate is generally linearly proportional to the [S] (i.e. at low [S] if you double [S] the V will double)

K_m

$K_m = (k_{-1} + k_2) / k_1$ = (rate of breakdown of ES)/(rate of formation of ES)

- K_m is **similar**, but not exactly equal to, a dissociation constant (K_d) for the ES complex
- If $k_{-1} >> k_2$, then $K_m » K_d$
- Due to this similarity to the expression for K_d, a **low value of K_m** is often interpreted as a **high affinity of the enzyme for the substrate**, and a large value for K_m is often interpreted as a weak affinity of the enzyme for the substrate
- K_m has units of molar concentration (just like the units for [S])

There is a mathematical treatment that allows for the determination of Km from the experimental V versus [S] data

- Consider the situation when the [S] being evaluated results in a value of V that is exactly $^1/_2$ of the maximum reaction velocity:

$V = V_{max}[S] / (K_m + [S])$

$^1/_2 V_{max} = V_{max}[S] / (K_m + [S])$

$^1/_2 = [S] / (K_m + [S])$

$K_m + [S] = 2[S]$

K_m = [S]

Thus, K_m equals the substrate concentration that results in exactly one half the maximum possible reaction velocity

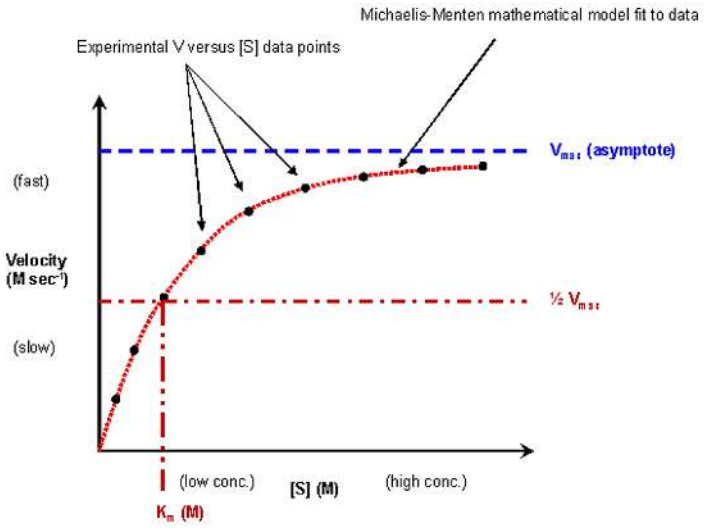


Figure 6.2.2: *K_m*

Lineweaver-Burke (the "*double reciprocal*" plot)

- The Michaelis-Menten equation can be rearranged by taking the reciprocal, to yield:

$$\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

- If X = 1/[S] and Y=1/***V*** then this is a linear equation with a slope of K_m/V_{max} and a Y intercept of $1/V_{max}$

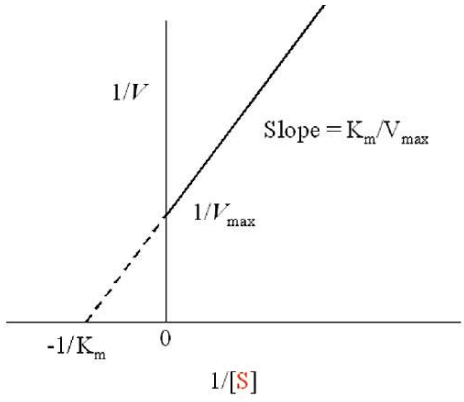


Figure 6.2.3: *1/S and1/V*

- Since the plot of 1/[S] versus 1/v data should be a straight line, it is easier to fit a linear function to the data in this form, and Vmax and K_m can be readily determined from the plot

Reversible Inhibition

There are two major categories of reversible inhibitors: **competitive** reversible inhibitors, and **noncompetitive** reversible inhibitors:

Competitive inhibitors

The inhibitor (**I**) competes with the substrate (**S**) for the enzyme active site (also known as the **S-binding site**). Binding of either of these molecules in the active site is a **mutually exclusive** event

- The substrate and inhibitor share a high degree of structural similarity. However, the inhibitor cannot proceed through the reaction to produce product.
- Increasing the concentration of substrate will **outcompete** the inhibitor for binding to the enzyme active site
- A competitive reversible inhibitor can be identified by its characteristic effects upon kinetic data

The expression for the Michaelis-Menten expression in the presence of a reversible competitive inhibitor is:

$V = V_{max}$ [S] / (K_m(1+[I]/K_i) + [S])

Where K_i is the actual EI complex dissociation constant

The effects of the reversible competitive inhibitor on the kinetics are as follows:

- If no inhibitor is present (i.e. if [**I**] = 0) then the equations are the same
- As inhibitor is added, the effect is to modify the apparent value of K_m. In particular, **the apparent K_m will be increased** by a value equal to **(1 + [I]/K_I)**. If K_m is increased, **the reaction velocity v will decrease**.
- Note that as [**S**] gets very large the value of the denominator is essentially equal to [**S**] and **v @ v_{max}**. Thus, the reaction velocity can be driven to vmax with a high enough substrate concentration

The diagnostic criteria for reversible competitive inhibition is that while the *apparent* Km is affected by addition of the inhibitor, the value of v_{max} does not change

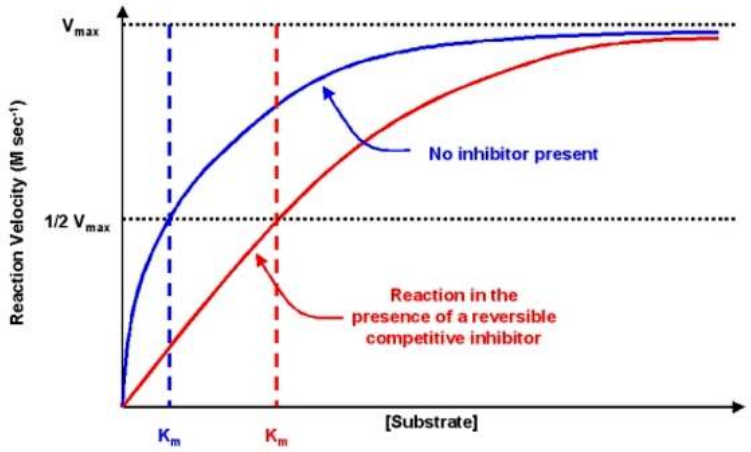


Figure 6.2.4: *Effect of reversible competitive inhibitor*

How is the Lineweaver-Burke double reciprocal plot affected by the presence of a reversible competitive inhibitor?

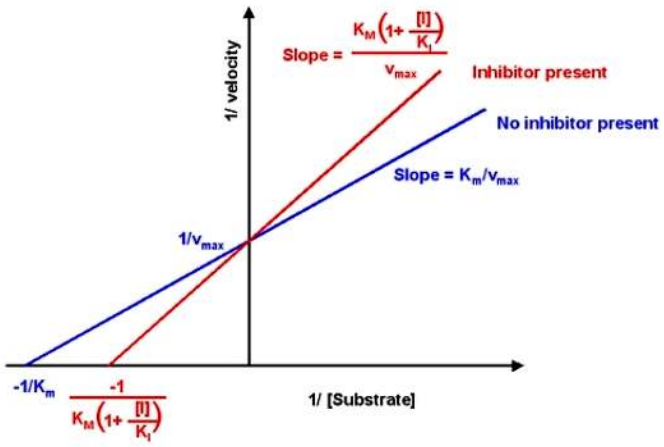


Figure 6.2.5: *Double reciprocal plot with reversible competitive inhibitor*

Noncompetitive Inhibitors

Noncompetitive inhibitors react with both E **and ES** (this is because the noncompetitive inhibitor does not bind at the same site in the enzyme as the substrate)

- Inhibition **cannot** be overcome by increasing the concentration of S
- The effect on kinetics is as if the enzyme were less active (**v_{max} is reduced**), but that the affinity for substrate is unaffected (**K_m remains the same**) since the substrate binding site is not occupied by the noncompetitive inhibitor.

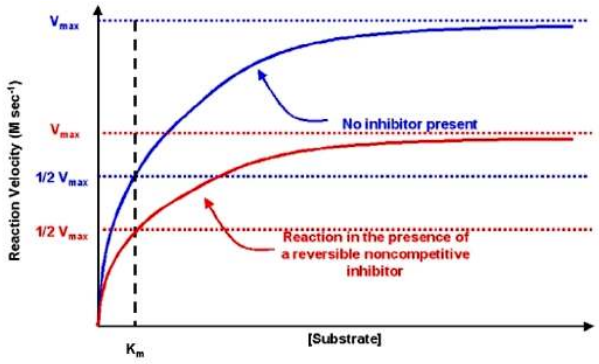


Figure 6.2.6: *Effect of reversible noncompetitive inhibitor*

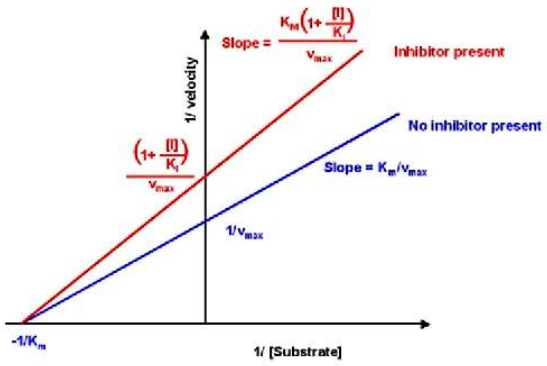


Figure 6.2.7: *Double reciprocal plot with noncompetitive inhibitor*

This page titled 10.5.2: Enzyme kinetics is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **6.2: Enzyme kinetics** by Michael Blaber has no license indicated.

10.5.3: Ligand binding

Molecular recognition is a key aspect of virtually all biochemistry, and involves:

- DNA replication
- Gene regulation
- Transcription of DNA into mRNA
- Translation of mRNA into proteins
- Enzyme function
- Molecular assembly
- Signal transduction
- Cell-cell communication
- Immunology

Intermolecular interactions, in the simplest example, involves **two molecules** recognizing and binding to each other:

- The **ligand**
- The **receptor**

These are broad terms, but the general properties of receptor and ligand are as follows:

- The ligand is typically, a small molecule, and it diffuses throughout the environment until it binds to a specific receptor
- The receptor is typically a large, relatively stationary molecule that contains a specific binding site for the ligand. Upon binding ligand, the structural and/or chemical properties of the receptor are typically altered

The interactions between receptor and ligand are typically non-covalent

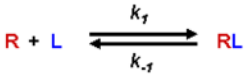
- There is a general molecular complementarity between the receptor and ligand
- Often, but not always, there is an entropic cost associated with binding of the ligand to receptor, but this is more than offset by a favorable enthalpy (i.e. the molecular complimentarity)
- The binding energy is typically utilized to cause a structural change associated with a molecular communication event

There are two general methods to study receptor/ligand interactions:

1. **Equilibrium thermodynamics**, and
2. **Association and dissociation kinetics**

Equilibrium ligand/receptor binding analysis

The two possible states of a ligand/receptor interaction, and the rate constants associated with their formation, are given as:



The **equilibrium association (formation) constant**, $\mathbf{K_f}$, is given as:

$$K_f = \frac{[RL]}{[R][L]}$$

- [RL] is the concentration of the complex, [R] is the concentration of free R at equilibrium, and [L] is the concentration of free Lat equilibrium
- K_f has units of M^{-1}
- A **large value for $\mathbf{K_f}$** means that the equilibrium favors the complex - there is a **high affinity of the receptor for the ligand**
- A **small value for $\mathbf{K_f}$** means that the equilibrium favors the separation of the receptor and ligand, and the receptor has **a low affinity for the ligand**

Just to make life difficult, the typical frame of reference when describing receptor affinity for ligand is to reference the **dissociation constant**, $\mathbf{K_d}$:

$$K_d = \frac{1}{K_f} = \frac{[R][L]}{[RL]}$$

- K_d has units of M
- A **small value for K_d** means that the equilibrium favors the complex - there is a **high affinity of the receptor for the ligand**
- A **large value for K_d** means that the equilibrium favors the separation of the receptor and ligand, and the receptor has **a low affinity for the ligand**

At **equilibrium**, the **rate of formation** of the RL complex is **equal** to the **rate of dissociation**:

Rate of formation of RL = $k_f[R][L]$

Rate of dissociation of RL = $k_{-f}[RL]$

At equilibrium: $k_f[R][L] = k_{-f}[RL]$

$$\frac{[R][L]}{[RL]} = \frac{k_{-f}}{k_f}$$

The left-hand expression is the definition of the dissociation constant, K_d, therefore:

$$K_d = \frac{[R][L]}{[RL]} = \frac{k_{-f}}{k_f}$$

The free energy expression and K_f and K_d

From the original expression of the complex formation:

$$R + L \underset{k_{-f}}{\overset{k_f}{\rightleftharpoons}} RL$$

$$K_f = \frac{[RL]}{[R][L]}$$

The standard state free energy change, ΔG^0, for the process (i.e. starting with 1M everything) would be given as:

$$\Delta G^0 = -RT\ln K_f$$

Since K_d is the inverse of K_f:

$$\Delta G^0 = -RT\ln K_f = RT\ln K_d$$

In this case, a negative value for DG^0 (indicating spontaneity) will occur when $K_d < 1.0$. This will happen when there is high affinity and the equilibrium favors the complex formation.

Practical relationships between [R], [L], K_d and [RL]

For the same concentration of added ligand and receptor, more complex formation will occur with a smaller value of K_d

Example 6.3.1

Receptor and ligand are added to a total concentration of 1.0×10^{-4}M. The K_d for the RL complex is 1.0×10^{-4}M. What is the concentration of [R], [L] and [RL] at equilibrium?

$$K_d = \frac{[R][L]}{[RL]} = 1.0 \times 10^{-4}$$

The added ligand, L, will be partitioned between free and bound forms:

$L_{total} = L + RL = 1.0 \times 10^{-4}$M

L = 1.0 x 10^{-4}M - RL

Similarly, the added receptor, R, will be partitioned between free and bound forms:

R_{total} = R + RL = 1.0 x 10^{-4}M

R = 1.0 x 10^{-4}M - RL

Substituting these values of L and R into the expression for K_d yields:

$$\frac{(1.0 \times 10^{-4} - RL)(1.0 \times 10^{-4} - RL)}{[RL]} = 1.0 \times 10^{-4}$$

$$(1.0 \times 10^{-4} - RL)(1.0 \times 10^{-4} - RL) = 1.0 \times 10^{-4}\ [RL]$$

$$1.0 \times 10^{-8} - 2.0 \times 10^{-4}RL + RL^2 = 1.0 \times 10^{-4}\ [RL]$$

$$RL^2 - 3.0 \times 10^{-4}RL + 1.0 \times 10^{-8} = 0$$

This is a quadratic with values a = 1, b = -3.0 x 10^{-4} and c = 1.0 x 10^{-8}. This yields two possible values for RL:

RL = 2.62 X 10^{-4}M or

RL = 3.82 X 10^{-5}

Since the maximum possible value for [RL] is 1.0 x 10^{-4} (i.e. given the starting concentrations of R and L, this is the most amount of RL that can be formed), the first result is not possible and **[RL] = 3.82 X 10^{-5}M**

Thus, 38.2% of the added receptor is in the complex formation (i.e. 3.82 X 10^{-5}M/1.0 x 10^{-4})x100%

Example 6.3.2

Same amount of added R and L, but this time the K_d for the RL complex is 1.0 x 10^{-6}M. What is the concentration of [R], [L] and [RL] at equilibrium?

$$\frac{(1.0 \times 10^{-4} - RL)(1.0 \times 10^{-4} - RL)}{[RL]} = 1.0 \times 10^{-6}$$

$$(1.0 \times 10^{-4} - RL)(1.0 \times 10^{-4} - RL) = 1.0 \times 10^{-8}\ [RL]$$

$$1.0 \times 10^{-8} - 2.0 \times 10^{-4}RL + RL^2 = 1.0 \times 10^{-6}\ [RL]$$

$$RL^2 - 2.01 \times 10^{-4}RL + 1.0 \times 10^{-8} = 0$$

his is a quadratic with values a = 1, b = -2.01 x 10^{-4} and c = 1.0 x 10^{-8}. This yields two possible values for RL:

RL = 1.11 X 10^{-4}M or

RL = 9.05 X 10^{-5}

Since the maximum possible value for [RL] is 1.0 x 10^{-4} (i.e. given the starting concentrations of R and L, this is the most amount of RL that can be formed), the first result is not possible and **[RL] = 9.05 X 10^{-5}M**

Thus, 90.5% of the added receptor is in the complex formation (i.e. 9.05 X 10^{-5}M/1.0 x 10^{-4})x100%

For the same concentration of added ligand and receptor, more complex formation will occur with a smaller value of K_d

Determination of dissociation (K_d) or binding (K_f) constants

The relationship between K_d, [R], [L] and [RL] is given by:

$$K_d = \frac{[R][L]}{[RL]}$$

- This is an equation with four variables, if you know three of them, you can solve for the fourth
- In principle, you can **solve for K_d** if you know the concentration of **free receptor** [R], **free ligand** [L] and **receptor/ligand complex** [RL] at **equilibrium**

To be able to solve for K_d, you need an assay that will provide the **molar concentrations** for each component. However, this is typically difficult to achieve. It is much more common to have an assay that will provide you with information regarding the **fraction** of added receptor (or ligand) that is **present in the free or complex** form.

- If [$\mathbf{R_t}$] is the **total** concentration of receptor in the sample, we know that it will be divided into two populations - bound and free:

$$[R_t] = [R] + [RL]$$

- The fraction of bound receptor sites is given by the ratio:

$$\text{Fraction of bound receptor molecules} = \frac{[RL]}{[R_t]}$$

- The fraction of bound receptors is known by the variable **a**, and **a** has a value between 0 and 1

$$\alpha = \frac{[RL]}{[R_t]}$$

- Substituting the value for $[R_t]$ in terms of [R] and [RL] given above yields:

$$\alpha = \frac{[RL]}{[R] + [RL]}$$

- The relationship between [RL] and K_d is given by the expression for Kd:

$$K_d = \frac{[R][L]}{[RL]}$$

$$[RL] = \frac{[R][L]}{K_d}$$

- This expression for [RL] can be substituted into the expression for a to yield:

$$\alpha = \frac{\frac{[R][L]}{K_d}}{[R] + \frac{[R][L]}{K_d}}$$

- Simplify by first dividing through by [R]:

$$\alpha = \frac{\frac{[L]}{K_d}}{1 + \frac{[L]}{K_d}}$$

- Then multiply by K_d:

$$\alpha = \frac{[L]}{K_d + [L]}$$

This equation has three variables:

1. The concentration of free ligand at equilibrium [L]
2. The dissociation constant, K_d, for the RL complex
3. The fraction of receptor that is in the bound form at equilibrium, a

If you know two of these variables, you can solve for the third (i.e. if the experimental assay quantifies [L] and a, you can solve for K_d)

The above equation can be manipulated further by substitution of the value of a in terms of [RL] and $[R_t]$:

$$\frac{[RL]}{[R_t]} = \frac{[L]}{K_d + [L]}$$

$$[RL] = \frac{[R_t]\,[L]}{K_d + [L]}$$

This equation has four variables:

1. The concentration of free ligand at equilibrium [L]
2. The dissociation constant, K_d, for the RL complex
3. The concentration of receptor that is in the bound form at eqilibrium [RL]
4. The total concentration of receptor in the sample

Usually, the ligand-binding experiment is setup so that [Rt] is held constant and [RL] is monitored as a function of [L]

This equation looks a lot like the Michaelis-Menten equation:

$$V = \frac{V_{max}\,[S]}{K_m + [S]}$$

Plotting V versus [S] in the M-M equation yields a rectangular hyperbola. Thus, **plotting [RL] versus [L]** will likewise yield a **rectangular hyperbola**

- The asymptote of the curve will yield the value of $[R_t]$
- The free ligand concentration [L] that results in one-half the maximum value of [RL] is equal to the K_d value

Yet another modification is to realize that if a equals the fraction of receptor bound in a complex (with a value $0 \leq \boldsymbol{\alpha} \leq 1.0$), then (**1 - α**) is the **fraction of free receptor** (i.e. **fraction of unbound receptor**)

$$\alpha = \frac{[RL]}{[R_t]}$$

$$1 - \alpha = 1 - \frac{[RL]}{[R_t]}$$

$$1 - \alpha = \frac{[R_t]}{[R_t]} - \frac{[RL]}{[R_t]}$$

$$1 - \alpha = \frac{[R_t] - [RL]}{[R_t]}$$

if $[R_t] = [R] + [RL]$

then $[R_t] - [RL] = [R]$

$$1 - \alpha = \frac{[R]}{[R_t]}$$

This value can be substituted into the three variable expressions above:

$$\alpha = \frac{[L]}{K_d + [L]}$$

$$1 - \alpha = 1 - \frac{[L]}{K_d + [L]}$$

$$\frac{[R]}{[R_t]} = 1 - \frac{[L]}{K_d + [L]}$$

$$[R] = [R_t] - \frac{[R_t][L]}{K_d + [L]}$$

$$[R] = \frac{[R_t]K_d + [R_t][L]}{K_d + [L]} - \frac{[R_t][L]}{K_d + [L]}$$

$$[R] = \frac{[R_t]K_d}{K_d + [L]}$$

This equation has four variables:

1. The concentration of free ligand at equilibrium [L]
2. The dissociation constant, K_d, for the RL complex
3. The concentration of receptor that is in the free form at eqilibrium [R]
4. The total concentration of receptor in the sample $[R_t]$

Usually, the ligand-binding experiment is setup so that [Rt] is held constant and [R] is monitored as a function of [L]

(Note:Although this looks like a M-M equation, **K_d is a constant**.)

The above derivations are called "**binding isotherms**" in reference to their ability to solve for Kd under equilibrium conditions (and the original equilibrium thermodynamic equation utilized constant temperature)

"Binding Isotherms"

$$[RL] = \frac{[R_t]\,[L]}{K_d + [L]}$$ Determine K_d from knowing [RL], $[R_t]$ and [L]

$$[R] = \frac{[R_t]\,K_d}{K_d + [L]}$$ Determine K_d from knowing [R], $[R_t]$ and [L]

Plotting data for various values of [L], $[R_t]$, and either [RL] or [R], and fitting to the appropriate function above, allows a more accurate determination of K_d (although a single experiment will provide an answer).

Due to the similarity of the [RL] versus [L] plot with the M-M plot, it is often the more desirable experimental setup. In particular, a **double-reciprocal plot** can be used (much like the double-reciprocal M-M plot) to more accurately determine K_d

$$\frac{1}{[RL]} = \frac{K_d + [L]}{[R_t]\,[L]}$$

$$\frac{1}{[RL]} = \frac{K_d}{[R_t]\,[L]} + \frac{1}{[R_t]}$$

$$\frac{1}{[RL]} = \frac{K_d}{[R_t]}\frac{1}{[L]} + \frac{1}{[R_t]}$$

$$Y = m * X + B$$

- If you plot 1/[RL] versus 1/[L] you will get a straight line whose slop is equal to $K_d/[R_t]$
- Y intercept will be equal to $1/[R_t]$. Since this value is fixed and is known, the value of the y intercept can serve as a check to make sure the experiment fits the model

An equilibrium dialysis experiment

A semi-permeable membrane can be used in a dialysis-based equilibrium experiment to determined Kd for a ligand-receptor pair.

- The membrane should be freely permeable as far as the ligand is concerned (usually it is a small molecule), but present a barrier to the receptor
- The experiment is established with a known concentration of receptor on one side, and a known concentration of ligand on the other
- The system is allowed to come to equilibrium
- The experiment requires an assay for the ligand

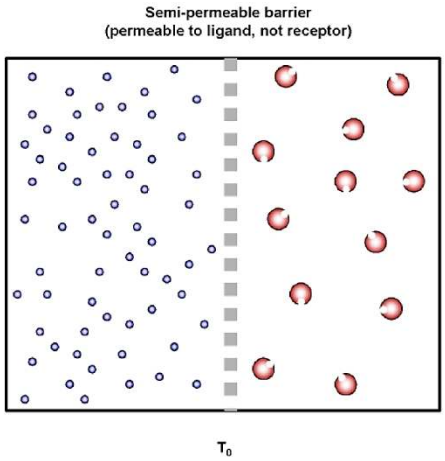


Figure 6.3.1: *Semipermeable barrier*

After equilibrium is reached the ligand concentration on both sides of the semi-permeable membrane is assayed:

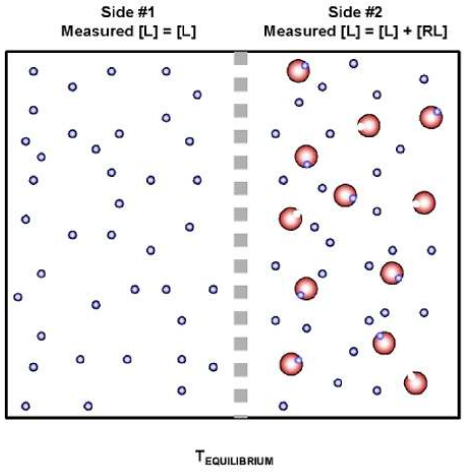


Figure 6.3.2: *Equilibrium concentration*

$[R_t]$ = known

[L] = Side#1[L]

[RL] = (Side#2[L] – Side#1[L])

[R] = [Rt] – [RL]

$$[RL] = \frac{[R_t]\,[L]}{K_d + [L]}$$

$$[R] = \frac{[R_t]\,K_d}{K_d + [L]}$$

- Since the ligand is unaffected by the membrane te ligand concentration on the side with no receptor is equal to the free ligand concentration [L] everywhere in the sample (i.e. on both sides of the membrane)
- The ligand concentration will be equal to the free ligand concentration [L] plus the ligand present in the receptor complex [RL] (in other words, there should be a higher concentration of ligand on the side with the receptor if the receptor has affinity for the ligand)
- The concentration of the receptor/ligand complex [RL] can be determined from the difference between these two values
- The total receptor concentration is a known value (experimenter determined at the start of the experiment, and does not change)
- Either binding isotherm can be used to solve for K_d (since [R] can also be determined)

This page titled 10.5.3: Ligand binding is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **6.3: Ligand binding** by Michael Blaber has no license indicated.

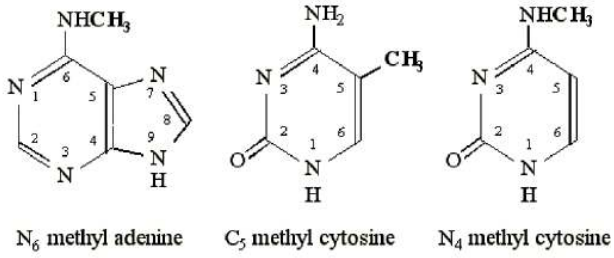
10.5.4: Restriction Mapping

The **restriction/modification system** in bacteria is a small-scale immune system for protection from infection by foreign DNA.

In the late 1960's it was discovered that *E. coli* contains enzymes that will **methylate** specific nucleotide bases in **DNA**

· Different strains of *E. coli* contained different types of these methylases

- Typical sites of methylation include the **N_6 position of adenine**, the **N_4 position of cytosine**, or the **C_5 position of cytosine**.



N_6 methyl adenine C_5 methyl cytosine N_4 methyl cytosine

Figure 6.4.1: *Methylation sites*

- In addition, only a fractional percentage of bases were methylated (i.e. not every adenine was methylated, for example) and these occurred at **very specific sites** in the DNA.
- ***A characteristic feature of the sites of methylation, was that they involved palindromic DNA sequences***.
- Here is an example from a particular E. coli strain R1:

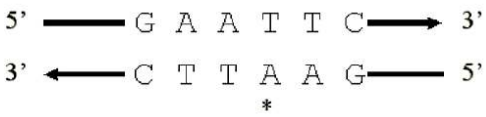


Figure 6.4.2: *Palindromic DNA*

(EcoR1 methylase specificity. Rubin and Modrich, 1977)

- In addition to possessing a particular methylase, individual bacterial strains also contained accompanying specific **endonuclease** activities.
- The endonucleases **cleaved at or near the methylation recognition site**.

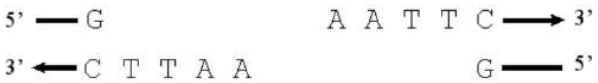


Figure 6.4.3: *Cleavage near methylation site*

- These specific nucleases, however, would **not** cleave at these specific palindromic sequences if the DNA was **methylated**.

Thus, this **combination** of a **specific methylase** and associated **endonuclease** functioned as a type of **immune system** for individual bacterial strains, protecting them from infection by foreign DNA (e.g. viruses).

- In the bacterial strain EcoR1, the sequence GAATTC will be methylated at the internal adenine base (by the EcoR1 methylase).
- The EcoR1 endonuclease within the same bacteria will not cleave the methylated DNA.
- Foreign viral DNA, which is not methylated at the sequence "GAATTC" will therefore be recognized as "foreign" DNA and willbe cleaved by the EcoR1 endonuclease.
- Cleavage of the viral DNA renders it non-functional.

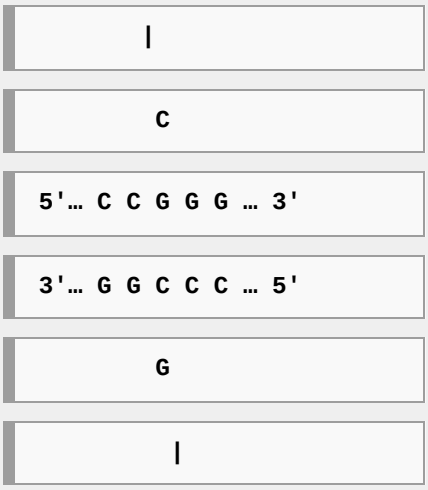
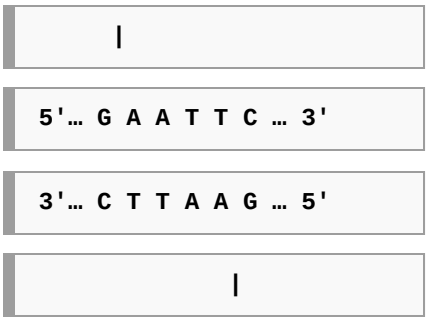
Such endonucleases are referred to as **"restriction endonucleases"** *because they restrict the DNA within the cell to being "self".*

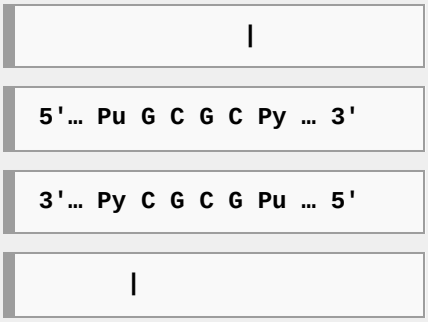
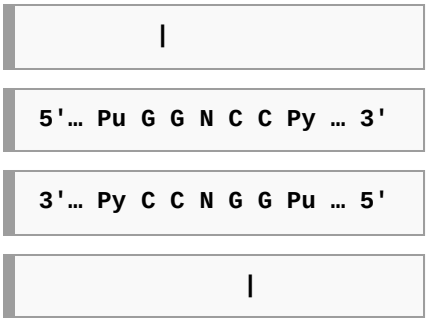
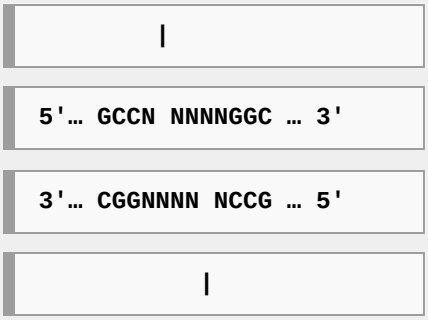
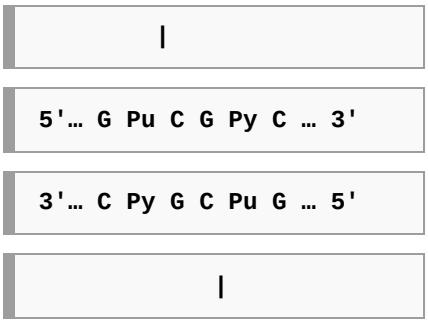
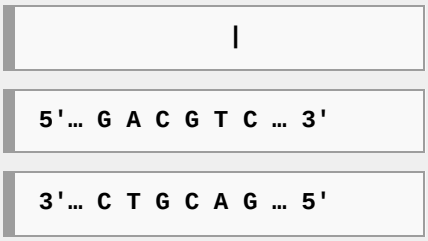
The combination of restriction endonuclease and methylase is termed the **"restriction-modification" system**.

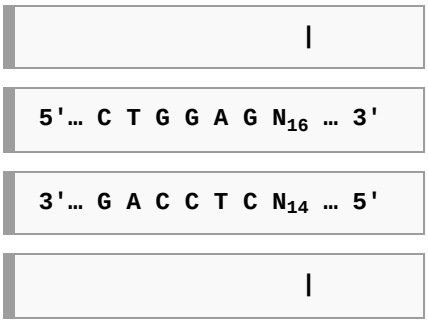
Since different bacterial strains and species have potentially different R/M systems, their characterization has made available **hundreds** of endonucleases with different sequence specific cleavage sites.

- They are one of the primary tools in modern molecular biology for the manipulation and identification of DNA sequences.

- Restriction endonucleases are commonly named after the bacterium from which it was isolated.

Name	Source	Recognition Sequence	Comments
Alu I	*Arthrobacter luteus*		"Four cutter". Leaves blunt ends to the DNA.
Bfa I	*Bacteroides fragilis*		"Four cutter". Leaves 5' overhang.
Nci I	*Neisseria cinerea*		"Five cutter". Middle base can be either cytosine or guanine. Leaves 5' overhang. Different recognition sites may have non-complementary sequences.
Eco R1	*Escherichia coli*		"Six cutter". Leaves 5' overhang. Behaves like a "four cutter" ('star' activity) in high salt buffer. $44 for 10,000 units.

Hae II	*Haemophilusaegyptius*		"Six cutter". Pu is any purine, Py is any pyrimidine. Leaves 3' overhang.
EcoO109I	*Escherichia coli*		"Seven cutter". Pu is any purine, Py is any pyrimidine, N is any base. Leaves 5' overhang. Different recognition sites may have non-complementary sequences.
Bgl I	*Bacillus globigii*		"Six cutter with interrupted palindrome". Leaves 5' overhang. Different recognition sites may have non-complementary sequences.
Bsa HI	*Bacillusstearothermophilus*		"Six cutter". Different recognition sites will be complementary.
Aat II	*Acetobacter aceti*		"Six cutter" with 3' overhang. Same recognition sequence as Bsa HI, but different cleavage position.

			
Bpm I	*Bacillus pumilus*		Non-palindrome, distal cleavage. Leaves 3' overhang. $50 for 50 units.
Not I	*Nocardiaotitidiscaviarum*		"Eight cutter". Leaves 5' overhang.
Bsm I	*Bacillusstearothermophilus*		"Weird". Leaves 3' overhang.

- The utility of restriction endonucleases lies in their specificity and the frequency with which their recognition sites occur within any given DNA sample.
- ***If there is a 25% probability for a specific base at any given site, then the frequency with which different restriction endonuclease sites will occur can be easily calculated (0.25^n):***

Specificity	Example	Frequency of Occurrence
Four base sequence	*Alu I*	1 Alu site in every 256 bases (0.25 Kb)
Five base sequence	*Nci I*	1 Nci I site in every 1024 bases (1.0 Kb)
Six base sequence	*EcoR I*	1 EcoR1 site in every 4,096 bases (4.1 Kb)
Seven base sequence	*EcoO109I*	1 EcoO109I site in every 16,384 bases (16.4 Kb)
Eight base sequence	*Not I*	1 Not I site in every 65,536 bases (65.5 Kb)

Thus, on average, any given DNA will contain an Alu I site every 0.25 kilobases, whereas a Not I site occurs once about every 65.5 kilobases.

- Not I is therefore a very useful enzyme for isolating large regions of DNA, typically in research involving genomic DNA manipulations.
- Alu I would be expected to digest a DNA sample into lots of little pieces.

The assortment of DNA fragments would represent a specific "**fingerprint**" of the particular DNA being digested. Different DNA would not yield the same collection of fragment sizes. Thus, DNA from different sources can be either matched or distinguished based on the assembly of fragments after restriction endonuclease treatment. **These are termed "Restriction Fragment Length Polymorphisms", or RFLP's**. This simple analysis is used in various aspects of molecular biology as well as a law enforcement and genealogy. For example, genetic variations that distinguish individuals also may result in **fewer or additional restriction endonuclease recognition sites.**

Gel Electrophoresis of DNA

The most common gel electrophoresis solid support matrix for DNA molecules is

- **agarose**and
- **acrylamide.**

DNA agarose gels

The electrophoretic migration rate of DNA through agarose gels is dependent upon ***four main parameters***:

1. The **molecular size** of the DNA. Molecules of linear duplex DNA travel through agarose gels at a rate which is inversely proportional to the log of their molecular weight.

M_r α 1/log (Mw)

Example: Compare molecular mass vs. expected migration rate:

Molecular Mass (Da)	**log (Molec. Mass)**	**1/log (Molec. Mass)** ***i.e. relative Mr***
100,000	5.0	0.20
50,000	4.7	0.21
10,000	4.0	0.25
5,000	3.7	0.27
1,000	3.0	0.33

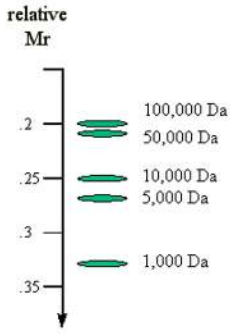


Figure 6.4.4: *Molecular mass and migration rate*

2. The **agarose concentration**. There is an inverse linear relationship between the logarithm of the electrophoretic mobility and gel concentration.

Agarose (%)	Range of separation of linear DNA (in kilobases)
0.3	60 - 5
0.6	20 - 1
0.7	10 - 0.8
0.9	7 - 0.5
1.2	6 - 0.4
1.5	4 - 0.2
2.0	3 - 0.1

3. The conformation of the DNA.

- closed circular DNA (***form-I***) - typically supercoiled (compact)
- nicked circular (***form-II***) - nick relaxes any supercoiling
- linear DNA (***form-III***)

These different forms of the *same DNA* migrate at different rates through an agarose gel.

- Almost always the **linear form** (form-III) migrates at the **slowest** rate of the three forms
- Supercoiled DNA (form-I) usually migrates the **fastest**

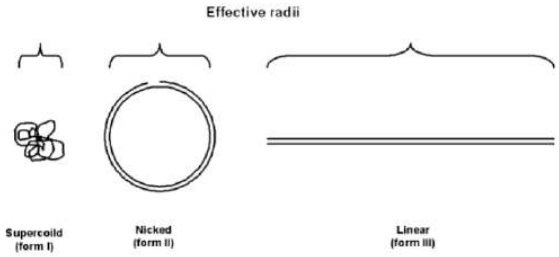


Figure 6.4.5: *Forms of DNA*

4. The applied **voltage**.

Other details:

- Typical value for running an agarose gel is **5 volts per cm** (length of gel).
- Agarose gels are usually poured and run horizontally
- *Finally, the DNA being an acidic molecule, migrates towards the positively charged electrode (cathode). DNA naturally has a constant charge to mass ratio, so no detergents need to be added (as with proteins)*

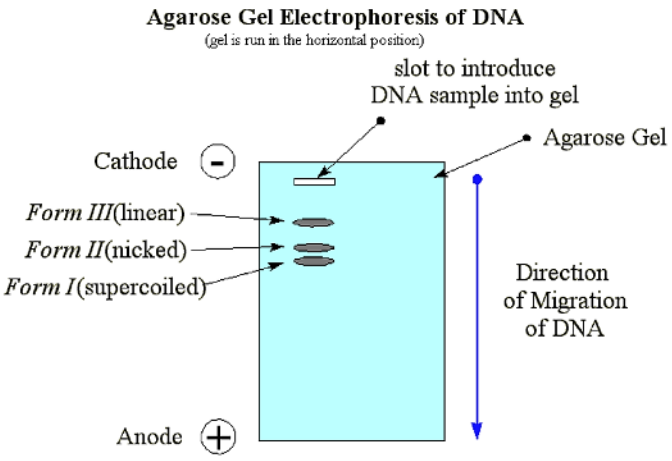


Figure 6.4.6: *Gel electrophoresis*

DNA acrylamide gels

- Acrylamide gels are useful for separation of **small DNA fragments**
  - typically oligonucleotides <100 base pairs.
  - These gels are usually of a *low acrylamide concentration* (<=6%) and contain the non-ionic *denaturing agent* Urea (6M).
    - The denaturing agent prevents secondary structure formation in oligonucleotides and allows a relatively accurate determination of molecular mass.

Staining of DNA

- The most convenient method to visualize DNA in gel electrophoresis is staining with the fluorescent dye **ethidium bromide**.

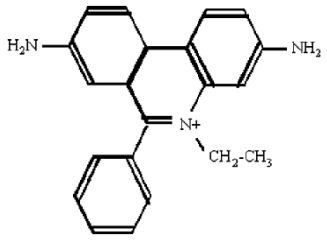


Figure 6.4.7: *Ethidium bromide*

- This compound contains a planar group that **intercalates** between the stacked bases of DNA.
- The orientation and proximity of ethidium with the stacked bases causes the dye to display an increased flourescencecompared to free dye (in solution).
  - U.V. radiation at 254 nm is absorbed by the DNA and transmitted to the bound dye.
  - The energy is re-emitted at 590 nm in the **red-orange** region of the spectrum.
- Ethidium bromide is usually prepared as a stock solution of 10 mg/ml in water, stored at room temp and protected from light.
- The dye is usually incorporated into the gel and running buffer, or conversely, the gel is stained after running by soaking in a solution of ethidium bromide (0.5 ug/ml for 30 min).
- The stain is visualized by irradiating with a UV light source (i.e. using a transiluminator) and photgraphing with polaroid film.
- The usual sensitivity of detection is better than 0.1 ug of DNA.

Because ethidium is a DNA intercalating agent, it is a powerful mutagen. Incorporation of ethidium in the DNA of living organisms (i.e. you and I) can cause (unwanted) mutations.

Combining restriction endonuclease digestion with gel electrophoresis of DNA: Restriction mapping

A given sequence of DNA (e.g. a gene) will have a specific sequence, and therefore, specific restriction endonuclease sites

- The number and location of such sites is a unique and predictable property for a given DNA molecule
- The fragmentation pattern (i.e. number and size of fragments after restriction endonuclease digestion) can be characterized by gel electrophoresis as a type of "DNA fingerprint"
- Any changes in the DNA sequence of a gene can result in the elimination of particular restriction sites, and conversely, create new ones:

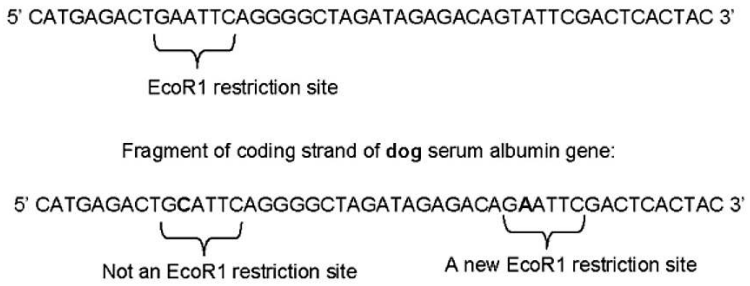


Figure 6.4.8: *Change in restriction sites*

- The gel electrophoresis fragment pattern for the human and dog albumin gene will be characteristically different from each other
- A sample of blood can potentially be identified as either human or dog by observing the **restriction fragment length polymorphism**
- Genetic differences between individuals can also be identified using restriction fragment length polymorphism analysis

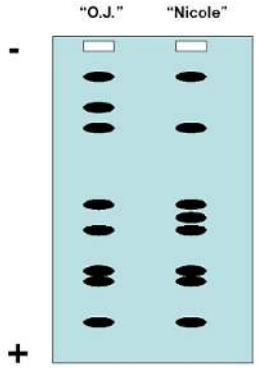


Figure 6.4.9: *Using RFLP to see genetic differences*

This page titled 10.5.4: Restriction Mapping is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **6.4: Restriction Mapping** by Michael Blaber has no license indicated.

10.5.5: Polymerase Chain Reaction (PCR)

Review

DNA replication is accomplished by the enzyme DNA polymerase which has the following characteristics:

1. DNA polymerase catalyzes extension of an oligonucleotide in the **5'→3' direction**. It cannot go in the opposite direction.
2. A **DNA template** (i.e. single strand oligonucleotide) is required. This is the information that is replicated (via Watson-Crick base pairing). If you want to replicate the "sense" strand of a DNA duplex, then the template is the anti-sense strand. Since DNA is a duplex, held together by non-covalent forces, we can separate the strands of the duplex (and obtain an appropriate template) by heat (or alkali) denaturation.
3. DNA polymerase can only extend an **existing oligonucleotide**, it cannot initiate replication. Thus, in addition to a template, DNA polymerase requires a primer oligonucleotide. DNA polymerase extends the primer in the 5'→3' direction.
4. Nucleotides incorporated into the nascent oligonucleotide must be **nucleoside triphosphates** (i.e. dNTP's). The energy released in the hydrolysis of the phosphate anhydride bonds is used in the formation of covalent linkages (incorporation of the a-phosphate into the phosphodiester backbone of the growing oligonucleotide)
5. Although DNA polymerases catalyze extension in the 5'→3' direction, some have the ability to "**proof-read**". "Proof reading" is the ability to recognize a misincorporation of a base, and the ability to backup and excise the incorrect base, and then continue on.

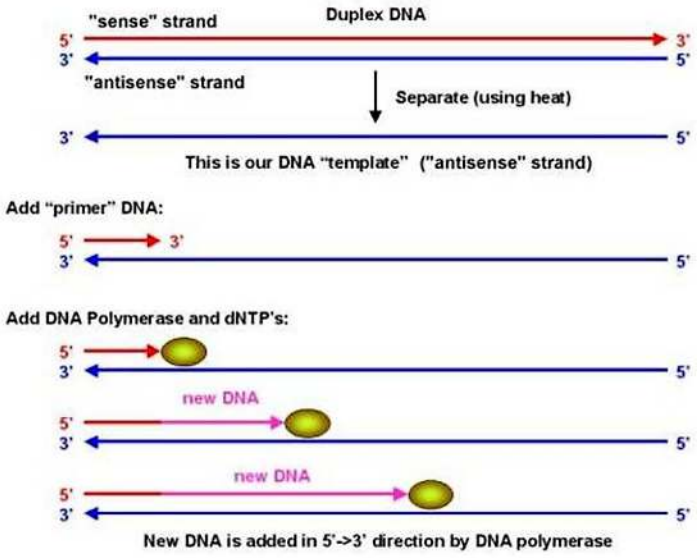


Figure 6.5.1: *DNA synthesis*

Polymerase Chain Reaction (PCR)

- PCR is an *in vitro* technique for the amplification of a region of DNA which lies **between two regions of known sequence**.
- PCR amplification is achieved by using oligonucleotide primers. These are typically short, single stranded oligonucleotideswhich are **complementary to the outer regions** of known sequence.

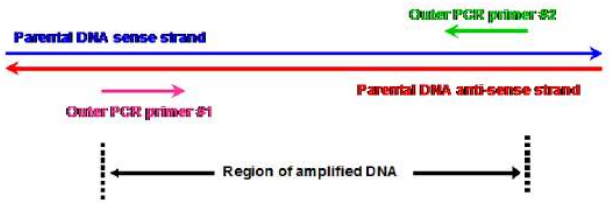


Figure 6.5.2: *PCR amplification*

The oligonucleotides serve as ***primers*** for DNA polymerase and each of the denatured strands of the parental DNA duplex serves as the ***template***.

This results in the synthesis of new DNA strands which are complementary to the parent template strands.

The steps of:

1. *Template denaturation*
2. *Primer annealing*
3. *Primer extension*

comprise a single "cycle" in the PCR amplification methodology.

- After each cycle the newly synthesized DNA strands can **serve as templates** in the next cycle (the PCR primers are typically added in substantial molar excess to the template DNA)

Summary of products at the end of each PCR cycle:

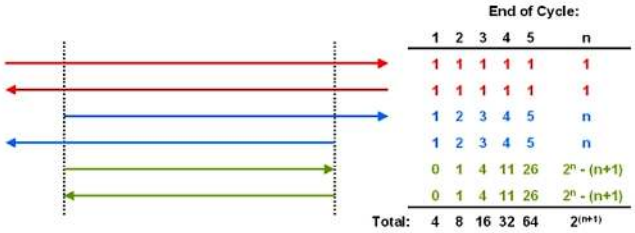


Figure 6.5.3: *PCR products*

The desired PCR product will be a **duplex of the defined length fragment**. The question is: how many will be produced?

· The expected ***amplification*** of the desired defined length product with respect to the original template concentration 'x' can thus be represented by the formula:

$[(2^n - (n + 1)) - (n + 1)]\ x$

or

$(2^n - 2(n + 1))$

(this is often abbreviated to a simple rule of thumb for the ***amplification***: $\mathbf{(2^n - 2n)\ x}$)

· The interpretation of this formula is that

- For a given number of cycles 'n' we make '2^n x' ***total possible duplexes***
- For a given number of cycles there will be '2(n+1) (or 2n in our approximation) x' duplexes which are formed from either the original template, or a fragment of indeterminate length, along with a fragment of defined length (and represent an undesired product)
- Thus, the total concentration of desired product (duplexes with a length defined by the PCR primers) will be $(2^n - 2(n+1))\ x$ (where x is the concentration of the original duplex)

The theoretical amplification value is never achieved in practice. Several factors prevent this from occuring, including:

1. Competition of complementary daughter strands with primers for reannealing (i.e. two daughter strands reannealing results in no amplification).

2. Loss of enzyme activity due to thermal denaturation, especially in the later cycles

3. Even without thermal denaturation, the amount of enzyme becomes limiting due to molar target excess in later cycles (i.e. after 25 - 30 cycles too many primers need extending)

4. Possible second site primer annealing and non-productive priming

Thermal cycling parameters

The thermal cycling parameters are critical to a successful PCR experiment. The important steps in each cycles of PCR include:

1. denaturation of template (typically performed at highest temp - 100°C)

2. annealing of primers (temperature is chosen based upon melting temperature of primer)

3. extension of the primers (performed at optimum for the polymerase being used)

A representative temperature profile for each cycle might look like the following:

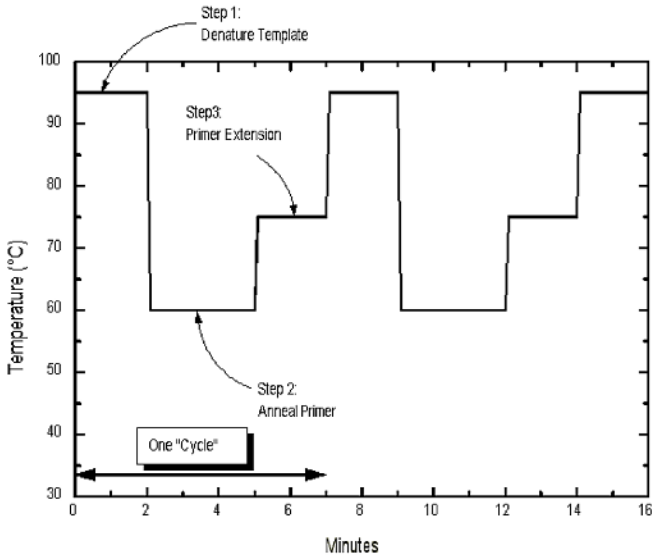


Figure 6.5.4: *Thermal cycling*

Buffers and $MgCl_2$ in PCR reactions

A typical reaction buffer for PCR would something like:

- 10 mM Tris, pH 8.3
- 50 mM KCl
- 1.5 mM $MgCl_2$
- 0.01% gelatin
- The $MgCl_2$ concentration in the final reaction mixture is usually between 0.5 to 5.0 mM, and the optimum concentration is determined empirically (typically between 1.0 - 1.5 mM). **Mg^{2+} ions**:
  - form a soluble complex with dNTP's which is essential for dNTP incorporation
  - stimulate polymerase activity
  - increase the Tm (melting temperature) of primer/template interaction (i.e. it serves to stabilize the duplex interaction

Generally,

- **low** Mg^{2+} leads to low yields (or no yield) and
- **high** Mg^{2+} leads to accumulation of nonspecific products (mispriming).

Choice of Polymerases for PCR

- One of the important advances which allowed development of PCR was the availability of thermostable polymerases.
- This allowed initially added enzyme to survive temperature cycles approaching 100 °C.
- Properties of DNA polymerases used in PCR

	Taq/Amplitaq®	Vent™	Deep Vent™	*Pfu*	*Tth*	ULTma™
95 °C half-life	40 min	400 min	1380 min	>120 min	20 min	>50 min
Extension rate (nt/sec)	75	>80	?	60	>33	?
Resulting ends	3' A	>95% blunt	>95% blunt	blunt	3' A	blunt
Stranddisplacement		+	+			
5'®3' exo	+				+	
3'®5' exo		+	+	+		+

Primers

Primer design

- Generally, primers used are 20 - 30 mer in length. This provides for practical annealing temperatures (of the high temperature regimen where the thermostable polymerase is most active).
- Primers should avoid stretches of polybase sequences (e.g. poly dG) or repeating motifs - these can hybridize with inappropriate register on the template.
- Inverted repeat sequences should be avoided so as to prevent formation of secondary structure in the primer, which would prevent hybridization to template
- Sequences complementary to other primers used in the PCR should be avoid so as to prevent hybridization between primers (particularly important for the **3' end** of the primer)
- If possible the 3' end of the primer should be rich in G, C bases to enhance annealing of the end which will be extended
- The distance between primers should be less than 10 Kb in length. Typically, substantial reduction in yield is observed when the primers extend from each other beyond ~3 Kb.

Melting temperature (Tm) of primers

- The Tm of primer hybridization can be calculated using various formulas. The most commonly used formula is:

(1) Tm = [(number of A+T residues) x 2 °C] + [(number of G+C residues) x 4 °C]

- Examples of T_m calculations

	G	A	T	C	T_m
15 mer	3	5	2	5	46
20 mer	6	5	4	5	62
30 mer	8	6	8	8	92

*If the annealing temperature is too high, **the primers will not anneal**. If it is too low, **mispriming** can occur at sites of similar DNA sequence to the intended primer binding site*

Analysis of the PCR experiment

- 25-30 cycles are common
- Major product should be a duplex DNA molecule whose 5' and 3' ends are defined by the two PCR primers
- Other products can, however, be produced. These will often represent products of secondary priming sites (mispriming) or misincorporation errors of the polymerase
- Thus, PCR product can be heterogeneous and must be separated and analyzed
- **Agarose gel electrophoresis**, in combination with ethidium bromide staining, is the most common method to separate and analyze PCR products

This page titled 10.5.5: Polymerase Chain Reaction (PCR) is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **6.5: Polymerase Chain Reaction (PCR)** by Michael Blaber has no license indicated.

10.5.6: Use of PC and internet for biochemical research

There are a variety of resources on the web related to biochemical research. These resources typically include general categories of:

- Databases of information
- Software for analysis of data
- Software for graphing and representing data
- Published reports and reviews (literature)

Databases of information

There are numerous repositories of information related to biological molecules and biochemistry. A few of these (along with some sample links) include:

- Databases of nucleic acid sequences, including commonly used plasmids.

The Entrez Nucleotide Database

New England Biolabs ™ Database of vectors and plasmids

- Protein sequence databases

Protein sequence database (Protein Information Resource)

Swiss-Prot knowledge database

- Protein structure database

The Protein Databank (PDB)

- Nucleic acid structure database

The Nucleic Acid Database

- Protein stability and thermodynamic database

Protherm Database

- Protein 2-D gel electrophoresis databases (proteomics)

EXPASY 2-D PAGE links

Software for analysis of data

Software can either be run on a local machine (i.e. your PC on your desktop), or on a server over the web. Sometimes, it is a combination of the two (i.e a Java™ applet that is served on a host machine, but requires Java software installed on your PC.

In general terms, if your data is expected to follow a particular mathematical model (e.g. Michaelis-Menten kinetics) then you needgeneral-purpose curve-fitting software. Links to non-linear least-square fit software include:

Datafit™ from Oakdale Engineering

LSM Least Squares Method curve fitting

Programs such as these allow you to simple write an equation, input your experimental values, and then the parameters of the equation (i.e. model) will be refined to give the best fit to the data. Thus, a single program can be used for a variety of applications (i.e. any situation where an equation is available that describes the behavior of the data)

Special purpose programs are available for specific applications.

Software for graphing and presenting data

In distinction to software for analysis (although sometimes combined in the same package) is software for graphing and representing data. The subtle point here is that after the data has been analyzed, you may need to prepare a report to communicate the results. In this case, there are other issues of data presentation - i.e. colors, line thicknesses, arrow styles, axis-breaks, fonts, legends, etc. that may need to be fussed with. Basic fitting software often does not include extensive presentation tools, and

similarly, presentation software often does not have robust and general purpose fitting tools. Examples of graphing and presentation software for scientific data are:

Origin™ from MicroCal™

KaleidaGraph from Synergy Software™

SigmaPlot™ from SPSS, Inc.

In addition to graphic representation of experimental data, some forms of data require three-dimensional representations. For example, biomolecular structures (e.g. RNA, DNA and protein molecules) are stored in databases (e.g. PDB) as a series of atomic coordinates, however, the data only really makes sense if you can convert such data into a representation of a three-dimensional molecule. Furthermore, understanding the structural information is possible by manipulation of such representations (e.g. rotations, translations, zooming in/out, etc.). Software for such manipulations includes:

Swiss PDB viewer

DS Viewer™ from Accelrys

Pymol

O

Furthermore, publication of figures of molecular structures often necessitate artistic embellishments - such as raytracing, shading, etc. The above programs can often output their figures in POV format, which allows such modifications using the following:

POV-RAY™

Literature databases

Scientific reports are published in various journals, meeting proceedings, and various books. Search engines are available to search electronic repositories of these materials. PubMed, a service of the National Library of Medicine, includes over 14 million citations for biomedical articles back to the 1950's. These citations are from MEDLINE and additional life science journals. PubMedincludes links to many sites providing full text articles and other related resources.

Here is a link to PubMed™

Additionally, it is also useful to be able to easily download, maintain, and format such references in papers or manuscripts that you have to prepare. Bibliography software allows you to do this quite easily. Examples of such software include ProCite, Reference Manager and Endnote (all from ISI ResearchSoft:

Endnote™ from ISI ResearchSoft™

Understanding DNA sequence information files

When requesting DNA sequence information there is a lot of additional information that is included in the file - in addition to the actual sequence. Here is an example of part of the file for the sequence of the plasmid pUC19:

```
Plasmid pUC19
```

```
Update 6/5/02
```

```
Features:
```

```
  469- 146      lacZ alpha CDS (start 469, complementary strand)
```

```
  519- 514      Plac promoter -10 sequence (TATGTT)
```

```
  543- 538      Plac promoter -35 sequence (TTTACA)

  575- 563      CAP protein binding site

  396- 452      multiple cloning site (EcoRI-HindIII)

 1455- 867      origin of replication (counterclockwise)

                  (RNAII -35 to RNA/DNA switch point):

 1273-1278        RNAI transcript promoter -35 sequence (TTGAAG)

 1295-1300        RNAI transcript promoter -10 sequence (GCTACA)

 1309-1416        RNAI transcript

 1419- 867        RNAII transcript (complementary strand)

 1434-1429        RNAII transcript promoter -10 sequence (CGTAAT)

 1455-1450        RNAII transcript promoter -35 sequence (TTGAGA)

 2486-1626      beta-lactamase (bla; amp-r) CDS

                  (start 2486, complementary strand)

 2486-2418      beta-lactamase signal peptide CDS

                  (start 2486, complementary strand)

      2521      bla RNA transcript start (complementary strand)

 2535-2530      bla promoter -10 sequence (GAGACA)

 2556-2551      bla promoter -35 sequence (TTCAAA)

puc19.seq  Length: 2686  June 5, 2002 13:46  Type: N  Check: 4141  ..

       1  TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG
```

```
      51  GAGACGGTCA CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG
```

```
     101  TCAGGGCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG CTTAACTATG
```

```
     151  CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGCG GTGTGAAATA
```

The actual DNA sequence starts towards the bottom of this file (it is truncated because it goes on for about 3000 bases). The start of the file is called the "header" and provides notes about features of the DNA sequence. For example, nucleotide bases 867 - 1455 comprise an origin of replication. It is listed as 1455 - 867 to communicate that the orientation of this "ori" is actually counterclockwise around the plasmid (plasmids are circular). There also is a gene for b-lactamase (bla; which provides for ampicillin resistance, or ampR selectable marker) that runs from basepair 1626 to 2486. It is also counterclockwise. Furthermore, the promoter for transcribing this gene is located from basepairs 2551 - 2521 (i.e. just "upstream" of the bla gene). The vector looks like this:

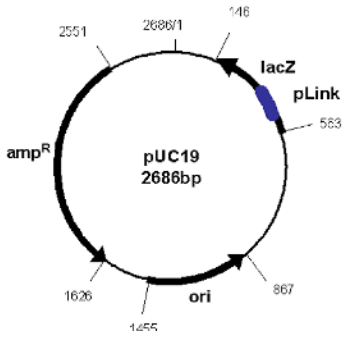


Figure 6.6.1: *pUC Vector*

The "start" of the sequence is basepair number 1, and is oriented at 12:00 (straight up). The sequence then runs clockwise around the plasmid. The plasmid diagram will use arrows to indicate the locations of the various features of the DNA, and the basepair numbers associated with the start and end locations of such features is also typically given on such figures.

This page titled 10.5.6: Use of PC and internet for biochemical research is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **6.6: Use of PC and internet for biochemical research** by Michael Blaber has no license indicated.

SECTION OVERVIEW

10.6: 4. Biotechnology 2

10.6.1: M13 Phage

10.6.2: M13 Phage Display Libraries

10.6.3: SELEX (Selective Evolution of Ligands by Exponential Enrichment)

10.6.4: Protein-Protein Recognition Probed Using a Yeast Transcriptional Activator System

10.6.5: Molecular Imprinting

This page titled 10.6: 4. Biotechnology 2 is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

10.6.1: M13 Phage

The bacteriophage known as "M13" forms the basis of cloning systems designed to easily introduce mutations into genes inserted into the phage genome. It also has been used in various "phage display" methodologies and "combinatorial" DNA and peptide libraries.

M13 infection and replication

M13 is a filamentous *bacteriophage* which infects *E. coli* host. The M13 genome has the following characteristics:

- Circular *single-stranded* DNA
- 6400 base pairs long
- The genome codes for a total of 10 genes (named using Roman numerals I through X)

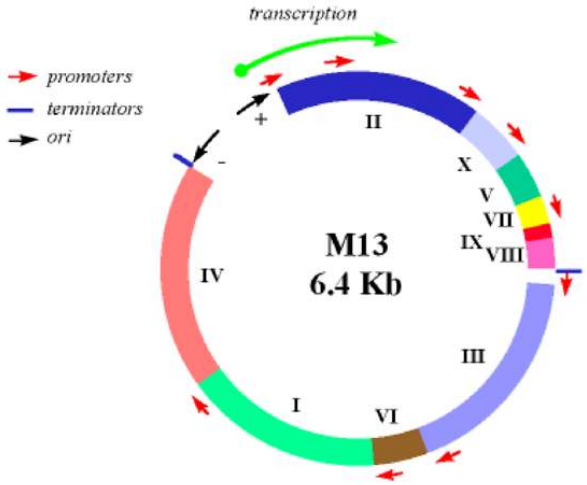


Figure 4.2.1: *M13 genome*

- *Gene VIII* codes for the major structural protein of the bacteriophage particles
- *Gene III* codes for the minor coat protein

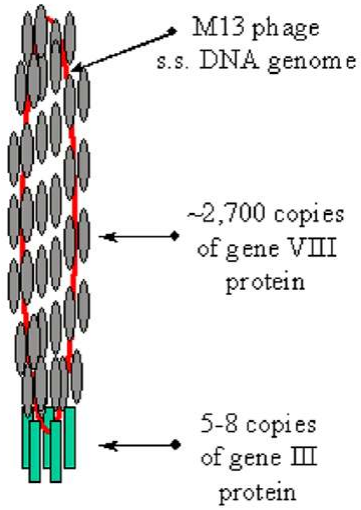


Figure 4.2.2: *Gene III and gene VIII*

- The gene VIII protein forms a tubular array of approx. 2,700 identical subunits surrounding the viral genome

- Approximately five to eight copies of the gene III protein are located at the ends of the filamentous phage (i.e. genome plus gene VIII assembly)
- Allows binding to bacterial "sex" pilus
  - Pilus is a bacterial surface structure of *E. coli* which harbor the "F factor" extrachromosomal element

Infection

- Single strand genome (designated '+' strand) attached to pilus enters host cell
  - Major coat protein (gene VIII) stripped off
  - Minor coat protein (gene III) remains attached
- Host components convert single strand (+) genome to double stranded circular DNA (called the replicative or "RF" form)
- Transcription begins
  - Series of promoters
    - Provides a gradient of transcription such that gene nearest the two transcription terminators are transcribed the most
  - Two terminators
    - One at the end of gene VIII
    - One at the end of gene IV
  - Transcription of all 10 genes proceeds in same direction

Amplification of viral genome

- Gene II protein introduces 'nick' in (+) strand
- Pol I extends the (+) strand using *strand displacement* (and the '-' strand as template)
- After one trip around the genome the gene II protein nicks again to release a completed (linear) '+' genome
  - Linear (+) genome is circularized
- During first 15-20 minutes of DNA replication the progeny (+) strands are converted to double stranded (RF) form
  - These serve as additional templates for further transcription
- Gene V protein builds up
  - This is a *single stranded DNA binding protein*
  - Prevents conversion of single (+) strand to the RF form
- Now get a buildup of circular single stranded (+) DNA (M13 genome)

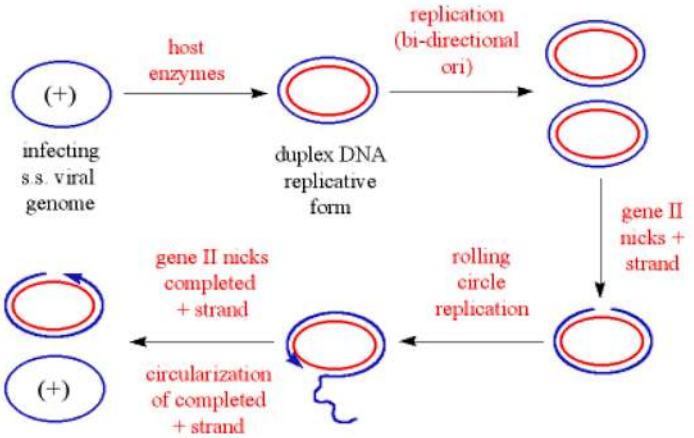


Figure 4.2.3: *Amplification of genome*

Phage packaging

- Major coat protein (Gene VIII) present in *E. coli* membrane
- M13 (+) genome, covered in ss binding protein - Gene V protein, move to cell membrane

- Gene V protein stripped off and the major coat protein (Gene VIII) covers phage DNA as it is extruded out
  - Packaging process is therefore *not linked to any size constraint* of the M13 genome
  - Length of the filamentous phage is determined by size of the DNA in the genome
  - Inserts of up 42 Kb have been introduced into M13 genome and packaged (7x genome size)
- ~8 copies of the Gene III protein are attached at the end of the extruded genome

Development of M13 into a cloning vector

M13 was developed into a useful cloning vector by inserting the following elements into the genome:

- a gene for the *lac* repressor (*lac I*) protein to allow regulation of the *lac* promoter
- the operator-proximal region of the *lac Z* gene (to allow for a-complementation in a host with operator-proximal deletion of the *lac Z* gene).
- a *lac* promoter upstream of the *lac Z* gene
- a polylinker (multiple cloning site) region inserted several codons into the *lac Z* gene

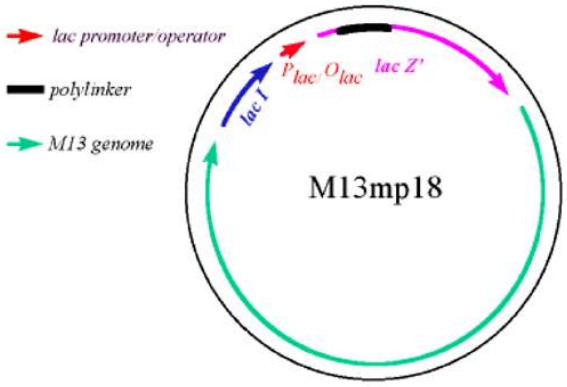


Figure 4.2.4: *Insertions into genome*

- The vectors were named according to the specific polyliner region they contained
- The vectors were typically constructed in pairs, with the polylinker regions in opposite orientations

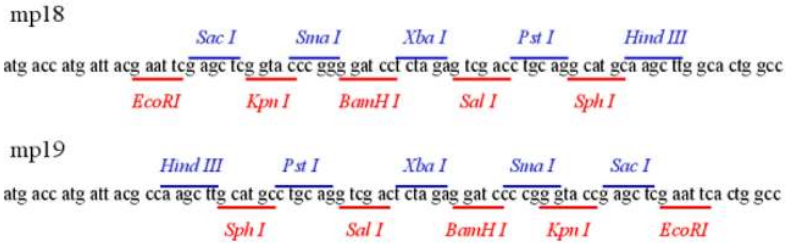


Figure 4.2.5: *Polylinker regions*

Cloning into M13mp vectors

- The RF (double stranded) form of the M13 phage can be isolated and treated just like any other plasmid
- The polylinker region can be "opened" using restriction endonucleases appropriate for accepting the fragment of interest
- The fragment is ligated into the plink region
- The availability of inverse oriented plink's (e.g. mp18, mp19) means that inserted fragments with non-complementary ends can be inserted in either orientation

Single stranded forms of the phage

The ability to isolated a single stranded form of the phage has advantages in both sequencing and mutagenesis.

- Single stranded DNA template can be read further than double stranded template

An efficient mutagenesis method (the "Kunkel" method) was developed using the single stranded form of the phage.

- The M13mp vector with insert is first grown in a mutant *E. coli* host (e.g. *CJ236*)which would occasionally incorporate uracil into the DNA instead of thymidine.
  - *E. coli* normally synthesizes an enzyme (uracil-N-glycosidase) that removes uracil residues in DNA. However, in *ung-* strains, the uracil is not removed
  - The level of uracil mis-incorporation into DNA is enhanced in strains which have a deficiency in dUTPase. This enzyme converts dUTP to dUDP, and therefore, in *dut-* strains the levels of dUTP are elevated and enhance misincorporation of dUTP into host DNA
  - The *E. coli* strain *CJ236* has a genotype which includes dut-/ung- features
- A mutagenic primer would be annealed to this single strand template (e.g. to produce a point mutation)
- The primer is extended using the four dNTP's, and is ligated to produce duplex DNA
- The duplex DNA is inserted into a different host *E. coli* (e.g. *JM101*) which recognizes and excises (degrades) uracil containing DNA (i.e. a strain which is *ung+*)
- The parent (wild type) strand is preferentially degraded and the mutagenic strand is replicated.
- The phage progeny typically have a high incidence (80-90%) of the desired mutation.

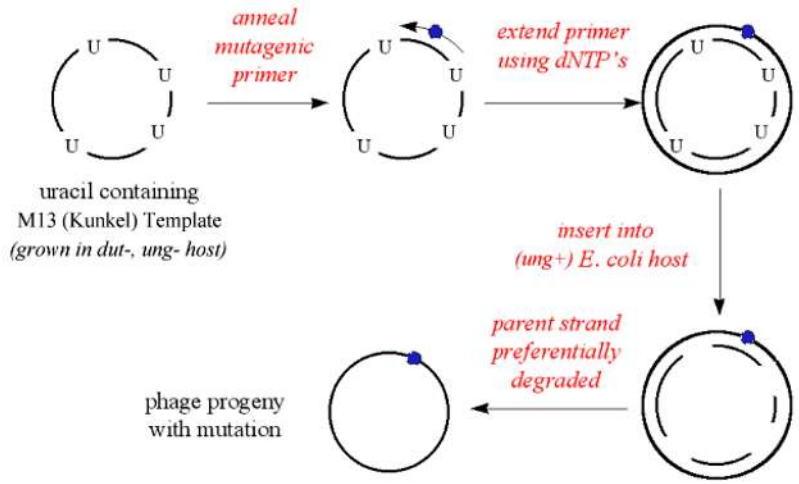


Figure 4.2.6: *Kunkel method*

This page titled 10.6.1: M13 Phage is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **4.2: M13 Phage** by Michael Blaber has no license indicated.

10.6.2: M13 Phage Display Libraries

Microbial systems developed for experimental molecular evolution

A protein or peptide is introduced at the amino-terminus of the major coat protein (Gene VIII) via PCR-based mutagenesis

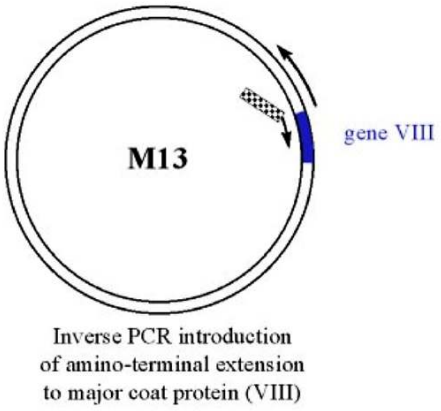


Figure 4.3.1: *Introduction of protein at gene VIII*

The linear PCR product is amplified to regenerate an intact circular viral genome whose gene VIII protein now has an amino terminal extension. The mutagenized M13 is introduced into *E. coli* cells. It is naked DNA (not packaged with viral coat proteins) so it is introduced into *E. coli* like a plasmid (i.e. via electroporation or using $CaCl_2$ competent *E. coli* cells).

- The extruded viral progeny will:
  - Be somewhat longer as the genome is larger but should be packaged o.k.
  - Express the mutant gene VIII (major coat) protein as part of the filamentous phage assembly

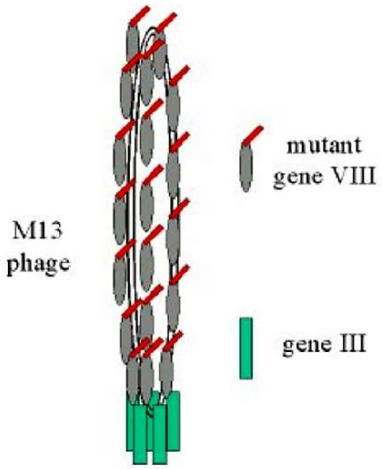


Figure 4.3.2: *Mutated M13 progeny*

Selection

The amino-terminal peptide may have affinity for a specific ligand, possibly an antibody. We can use this binding affinity to purify the mutant phage. We can can construct a chromatography column where the chromatography matrix has attached to it the ligand of interest. If we pass our phage through this column, the mutant phage containing the mutant gene VIII protein will bind to, and be immobilized, on the column

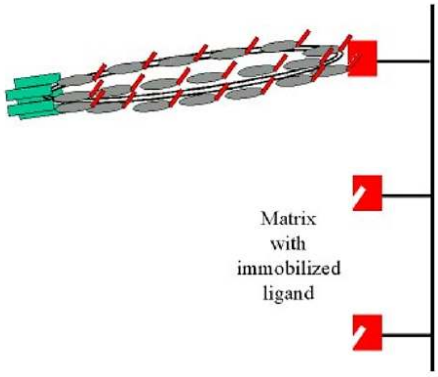


Figure 4.3.3: *Immobilized ligand*

- Any wild-type phage will not bind, and will pass through the column
- The bound phage can be eluted and used directly to reinfect *E coli* to propagate viruses with the mutant gene VIII protein

The mutant gene VIII protein which has been purified carries along with it the DNA which codes for itself. In other words, we have specifically selected for the DNA which codes for the gene VIII of interest (i.e. the mutant)

Changing binding specificity

Suppose we have a slightly different ligand attached to the matrix…



Our phage with mutant gene VIII protein will not bind very well to this different ligand. If we introduce the appropriate amino acid changes into our mutant gene VIII protein, we may be able to improve the binding affinity (specificity) and get the phage to bind to the new ligand

We do not know what specific amino acid changes to make, so we will introduce random changes into the amino terminal extension of the gene VIII protein

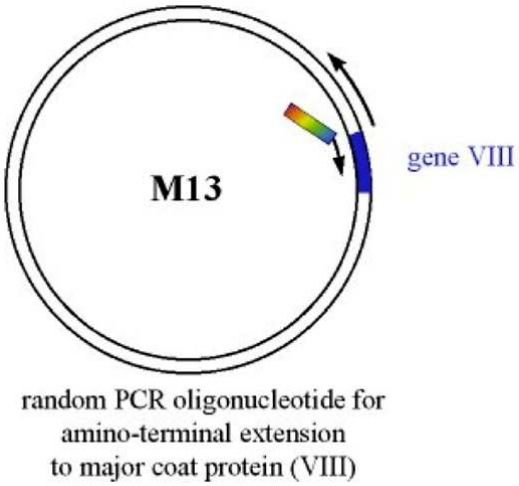


Figure 4.3.4: *Random changes in amino-terminal extension*

- After PCR and ligation, this will produce a **library** of phage genomes, each with a potentially *different* sequence for the amino terminal extension of the gene VIII protein
- Generally speaking, when these are introduced into *E. coli* the efficiency is typically so low that, at most, each successful transformation results in only one phage genome per *E. coli*

- Progeny phage from our library will, therefore, have only one type of gene VIII protein per phage (a single phage will have the gene VIII protein which it's DNA codes for).
- If we pass the collection of progeny phage from our library over our ligand column, we will selectively bind those phage whose mutant gene VIII protein has affinity for the ligand

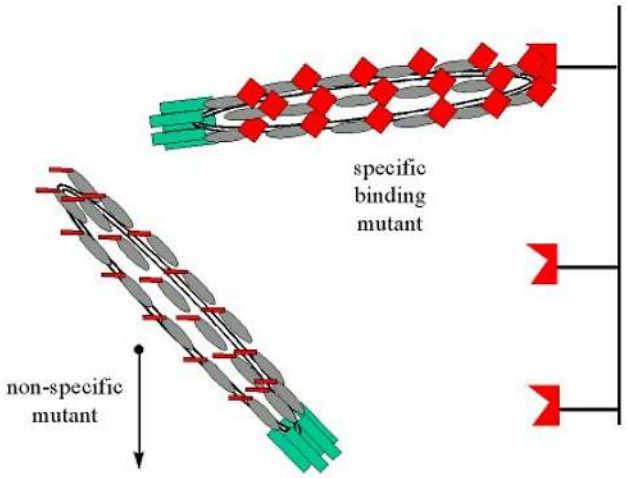


Figure 4.3.5: *Specific binding*

- If we isolate the bound phage we can re-infect *E.coli*, and isolate the progeny phage

This will result in a second library which will be enriched for mutant gene VIII sequences with the desired ligand binding specificity

- In this first selection we may have many other phage which might be weak, or generally non-selective, ligand binders. So, the process is repeated until we have a library of specific high-affinity mutants (this is sometimes called "Bio-mining" or "Bio-panning")
- **We can then sequence the DNA and find out what sequence(s) in the gene VIII mutant provide high binding specificity to our new ligand**

Practical limitations

- Over what length of DNA/protein can we randomize?

Example: a random stretch of 10 amino acids

- 20^{10} possible different unique polypeptide sequences. Each amino acid is 110 g/mol or 1.8×10^{-22}g per amino acid. This would mean that a complete random library of 10mer peptides would have a mass of 1.8×10^{-9} g.

Example: a random stretch of 20 amino acids

- 20^{20} possible different unique polypeptide sequences. Each amino acid is 110 g/mol or 1.8×10^{-22}g per amino acid. This would mean that a complete random library of 20mer peptides would have a mass of **18.9 Kg!**

This does not even consider the significantly larger mass of the virus particle of which it would be a part. This type of calculation demonstrates that generating random DNA sequence libraries is limited to very short stretches of about half a dozen or so codon positions.

Random Mutagenesis

We could randomly introduce all four bases at each position (NNN) while trying to synthesize a mutagenic random codon, but what would this give us?

Leu, Arg, Ser (6 codons/64)	9.4%
Val, Pro, Thr, Ala, Gly (4 codons/64)	6.3%
Ile (3 codons/64)	4.7%
Phe, Tyr, His, Gln, Asn, Glu, Asp, Cys (2 codons/64)	3.1%
Met, Trp (1 codon/64)	1.6%
Stop (3 codons/64)	4.7%

- Thus, not all amino acids are present in equal amounts, and we will have 4.7% stop codons
- We can improve this by using all four bases at the first two positions, and an equal mix of G and C bases at the third (wobble) position. This would result in 32 possible codons and the following distribution of amino acids and codons:

Phe, Tyr, Cys, Trp, His, Gln, Ile, Asn, Met, Lys, Asp, Glu, Stop (1 codon/32)	**3.1%**
Gly, Val, Ala, Pro, Thr (2 codons/32)	**6.3%**
Leu, Arg, Ser (3 codons/32)	**9.4%**

Another general problem with the above method is that of **non-specific** binding

- This is due in part to the large number of mutant gene VIII proteins (~2500) on the virus surface.
- Ligand binding may be due to the interaction of two or more gene VIII proteins contributing to binding the ligand (i.e. "polyvalent" binding)
- In this case, the isolated mutant peptide may show weak or no binding affinity at all

How can we minimize the number of mutant proteins per phage particle?

Approach #1 - Mutate gene III protein instead of gene VIII

- While there are ~2,500 copies of the gene VIII (major coat protein) per phage, there are 5 copies of the gene III (minor coat protein) per phage

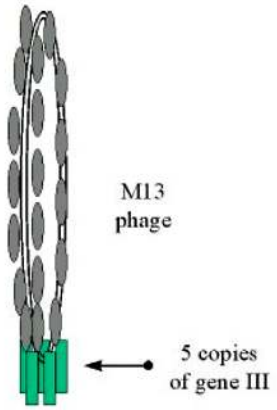


Figure 4.3.6: *Mutating gene III*

- We can introduce the mutagenic sequence to the amino-terminal of the gene III protein and thereby limit the number of copies on the phage particle

- this may still result in polyvalent binding

Approach #2 - Supplement with wild-type gene III protein during phage assembly

- If the M13 phage contains a second copy of gene III which we do not mutate then when the phage assembles (as it extrudes through the outer membrane) the five gene III proteins it picks up will be a mixture of mutant and wild type (in roughly equal amounts). In this case, the progeny phage will have, on average, 2-3 copies of the mutant gene III protein
- If we can increase the expression of the wild type gene III protein, to the point where it is present in four-fold excess over the mutant, then the progeny phage will, on average, contain 1 copy of the mutant gene III protein (and ligand binding will, by definition, be mono-valent)

How can we do this?

- We can put a strong promoter in front of the wild type gene III
- We could place the wild type gene III near one of the terminators (after gene IV, for example). This position would result in higher expression of the wild type gene due to the cumulative effects of the upstream promoters

Variation

Expression of mutagenic protein as a fusion of an outer membrane protein of *E.coli* itself

- We can attach our mutagenic sequence to a gene for an outer membrane protein (e.g. *OmpT, LamB*)
- The surface of the *E. coli* cell itself displays the mutagenic peptide
- Outer membrane proteins may be present at a level of 10,000 molecules or more
- Our ligand column will selectively bind specifically binding *E. coli* cells. These can be eluted and cultured and the process repeated to select for the mutant sequence with binding affinity

Expression of mutagenic protein as a fusion with a DNA binding protein

- We can attach our mutagenic sequence to a gene for a protein which binds DNA (e.g. *lac* repressor)
- The mutagenic lac repressor will be encoded by a plasmid which itself contains two *lac* operator sequences
- The mutant repressor molecule expressed by the plasmid will bind strongly to the *lac* operator regions on the very same plasmid
- The cells are lysed (the repressor remains bound to its associated plasmid DNA) and the ligand binding step is performed to enrich for ligand binding sequences
- The associated plasmid DNA is isolated and transformed back into *E. coli* for amplification and another round of selection

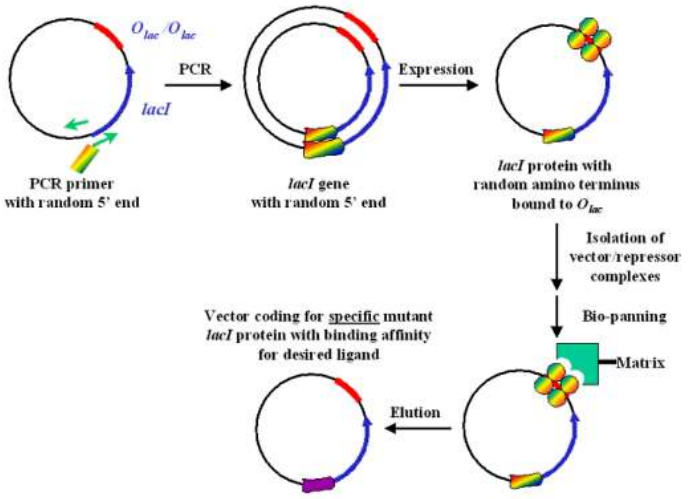


Figure 4.3.7: *Fusing mutagenic protein and binding protein*

This page titled 10.6.2: M13 Phage Display Libraries is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **4.3: M13 Phage Display Libraries** by Michael Blaber has no license indicated.

10.6.3: SELEX (Selective Evolution of Ligands by Exponential Enrichment)

Tuerk and Gold, *Science* (1990) **249**:505-510

Phage T4 genome encodes a DNA polymerase (T4 DNA pol)

The mRNA for T4 DNA pol contains a sequence which has binding affinity for T4 DNA pol. This sequence overlaps the Shine/Dalgarno sequence and binding of T4 DNA pol prevents the mRNA from being transcribed (and producing more T4 DNA pol). Thus, the expression of T4 DNA pol is self-regulating (***autogenous regulation***). The structure of the mRNA in this region forms a stem-loop structure



Figure 4.4.1: *T4 DNA polymerase mRNA*

The experimental design:

In simple terms, generate a library of mRNA molecules with a random nucleotide sequence in the loop region. This library will have a size of 4^8 or 65,536 uniquely different sequences (if only the loop region is randomized). Identify those sequences with high affinity for T4 DNA pol and determine their sequence.

Template Construction

A "synthetic gene" duplex DNA template is constructed from five primers:

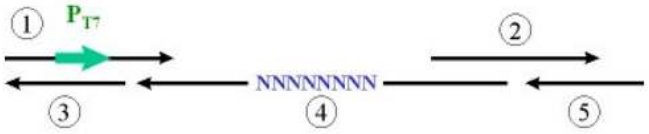


Figure 4.4.2: *Template construction*

- The synthetic gene is 110 nucleotides in length. It is composed of oligonucleotides #3, #4 and #5. Oligonucleotides #1 and #2 can be thought of as "bridging" oligos which allow #3, #4 and #5 to be ligated
- Oligonucleotide #4 contains the 5' end of the T4 DNA Pol gene corresponding to the mRNA Shine/Dalgarno sequence and the T4 DNA pol binding step/loop. It is synthesized to contain ***random bases*** at the 8 base long loop structure of the T4 DNA pol binding domain.
- Oligonucleotide #1 contains the sequence for T7 RNA polymerase promoter region. Transcription can be driven from this promoter by addition of T7 RNA polymerase and ribonucleotides.

in vitro transcription

- The 110 nucleotide gene (double-stranded DNA form) can be transcribed *in vitro* by T7 RNA pol
- Transcription produces a 92 nucleotide long RNA molecule. There will be a library of potentially 65,536 unique RNA molecules

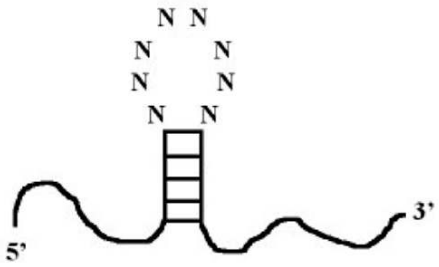


Figure 4.4.3: *In vitro transcription*

Selection by binding to T4 DNA pol

Those RNA transcripts with a sequence which confers binding specificity for T4 DNA pol are ***selected for*** by incubating the RNA library with T4 DNA pol which has been immobilized on nitrocellulose filters. The filters are washed to remove non-specifically bound RNA molecules. Tight (specific) binding RNA molecules are eluted from the T4 DNA pol protein and are collected

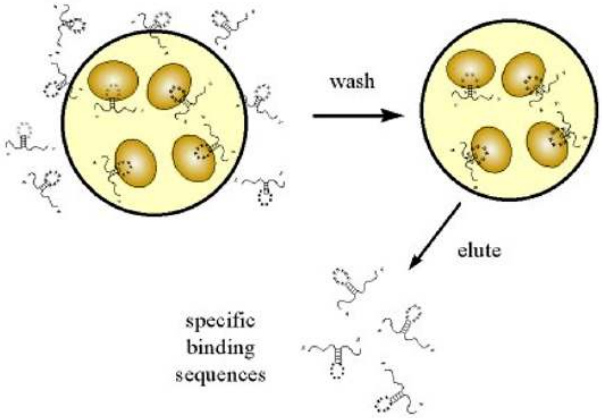


Figure 4.4.4: *Specific binding*

cDNA construction

The eluted RNA molecules are converted to single strand cDNA using oligonucleotide #5 as a primer, and adding Reverse transcriptase and dNTP's. Duplex DNA is produced from the single strand cDNA by PCR using oligonucleotides #1 and #5.

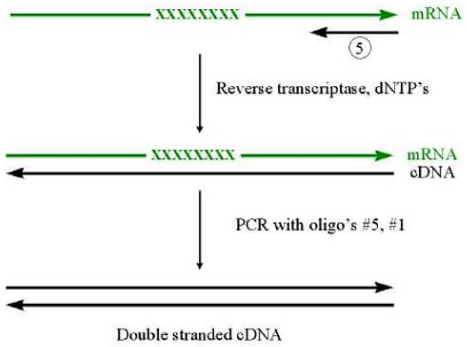


Figure 4.4.5: *cDNA Construction*

In vitro transcription

- In vitro transcription produces a (smaller) library of RNA molecules enriched for sequences with binding affinity for T4 DNA pol
- the process of selection and enrichment is continued until a collection of high affinity binding sequences has been produced

Experimental results

1. Library construction

- The original "synthetic gene" library was sequenced *en mass*
- A "ladder" of bases at the random position indicated that the library started out being more or less a completely random collection of sequences in this region (i.e. there did not appear to be any sequence bias in the collection of molecules)

2. Samples from the sequential rounds of selection and amplification were saved for sequencing analysis

- The results indicated that as the selection and amplification process continued certain bases were observed at specific positions in the 8 base mutagenized region

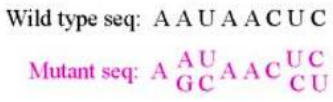


Figure 4.4.6: *Mutant sequence*

The wild type sequence could be found within this heterogenous mixture of tight binding sequences. 20 clones from this final library were sequenced.

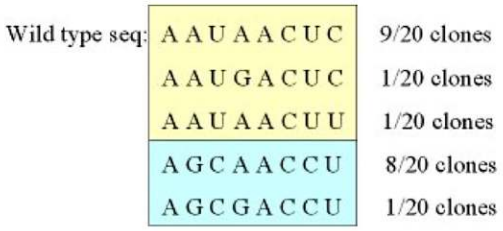


Figure 4.4.7: *Mix of tight binding sequences*

- There were essentially only two different sequences present: the known wild type sequence and another different sequence (which varied at four positions)
- Both had similar tight binding characteristics (the minor sequences identified exhibited weaker binding)
- This alternative sequence may have a different stem-loop structure than the wild-type

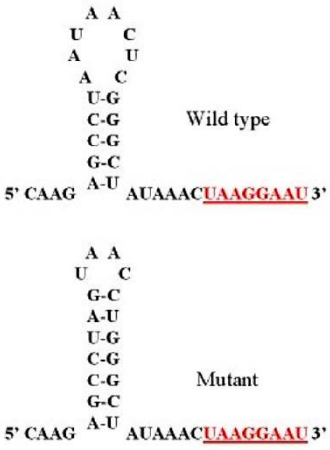


Figure 4.4.8: *Change in stem loop structure*

Conclusions

1. The SELEX method successfully identified the wild type sequence and an alternative (previously unknown) sequence with high affinity T4 DNA pol binding properties
2. The results suggest that the sequence of the wild type translational operator is pretty much optimized for binding (unable to improve further?)
3. The SELEX method should simplify the study of interactions between
   - transcriptional activators and repressors and transcriptional complexes at promoter sites
   - replication accessory proteins and DNA pols at origins of replication
   - ribosomes and translational repressors at ribosome binding sites
4. Results may be relevant to DNA/protein binding interactions in certain cases (i.e. if interactions do not involve 2' hydroxyl group and if a structure which DNA can adopt is involved)
5. Instead of beginning with all possible sequences, start with a smaller library and combine replication with error prone polymerases to generate additional sequences

---

This page titled 10.6.3: SELEX (Selective Evolution of Ligands by Exponential Enrichment) is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **4.4: SELEX (Selective Evolution of Ligands by Exponential Enrichment)** by Michael Blaber has no license indicated.

10.6.4: Protein-Protein Recognition Probed Using a Yeast Transcriptional Activator System

Fields, S. and Ok-kyu, S. *Nature* (1989) **340**:245-246

Background

The yeast genome contains *Upstream Activator Sequences* (UAS's)

UAS are located 5' to the coding region of a gene and are regulatory regions - they bind transcriptional activator proteins. These transcriptional activator proteins, when bound to UAS, stimulate transcription (i.e. they may contain binding sites for RNA polymerase

Yeast gene, protein nomenclature

Wild type genes are designated with capital letters in italics (e.g. ***GAL4***). Mutant alleles of the genes are indicated in lowercase italic (e.g. ***gal4***) and encoded proteins are indicated by roman type, with the first letter capitalized (e.g. **Gal4**)

Growth of yeast in galactose containing media

Incubation of wild type yeast in galactose results in >1,000 fold increase in the mRNA level for enzymes involved in galactose metabolism. This increase in mRNA levels is not observed in *gal4* mutants. The Gal4 protein is not one of the enzymes involved in galactose metabolism, rather, it a appears to be a *transcriptional activator protein* which specifically activates the transcription of genes involved in the galactose metabolic pathway. Gal4 protein binds to a specific 17 nucleotide long region of DNA located in the 5' region of genes involved in galactose metabolism. These regions are termed UAS_{GAL} Binding of Gal4 to UAS_{GAL} increases transcription from the nearby promoter

Gal4 Protein

- Single polypeptide chain which contains two domains:

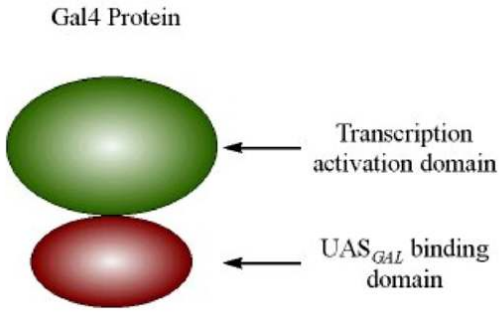


Figure 4.5.1: *Gal4 protein domains*

These domains are functionally separable in the polypeptide chain (Figure 4.5.2). The amino-terminal (1-74) domain by itself can bind to UAS_{GAL} sequences but cannot activate transcription. The carboxyl-terminal (738-881) domain contains the activating region but cannot activate transcription because it fails to localize to the UAS_{GAL} region (i.e. near the promoter region)

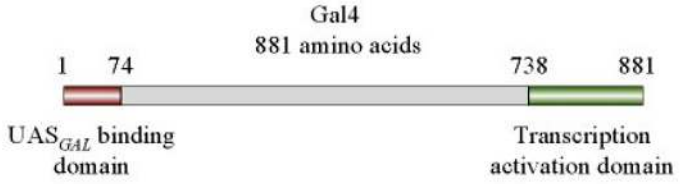


Figure 4.5.2: *Gal4 polypeptide chain*

Imagine we have two proteins (X and Y), which normally form a stable complex (XY)

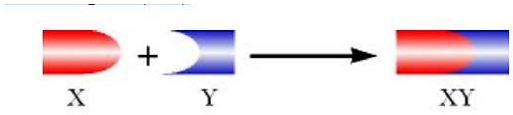


Figure 4.5.3: *Complex*

If we make a Gal4(1-74)- X fusion protein and a Y -Gal4(738-881) fusion protein we can potentially form a functional Gal4 transcription activator (i.e. a Gal4 activator region in complex with a UAS_{GAL} region

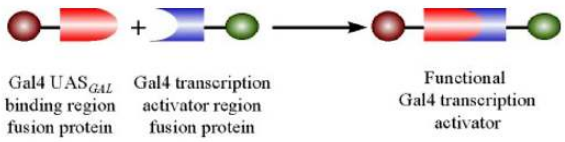


Figure 4.5.4: *Formation of Gal4 transcription activator*

This fusion protein complex can act as a functional transcriptional activator for the galactose metabolic enzymes

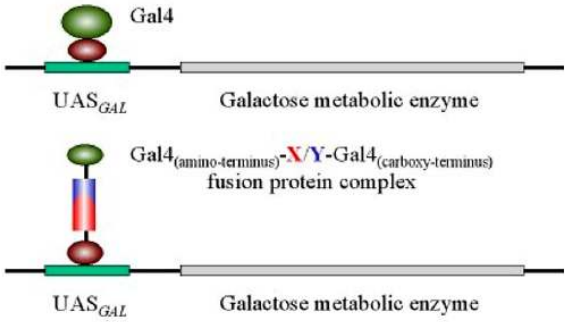


Figure 4.5.5: *Protein complex acting as transcription activator*

To test this hypothesis, the authors used the following system

- The **protein complex** involved the two proteins SNF1 and SNF4. The SNF1 protein is a serine-threonine specific protein kinase, and the SNF4 protein is known to be physically associated with SNF1 and is essential for function
- 5 different gene constructs involving Gal4 and SNF1 and SNF4 were made:

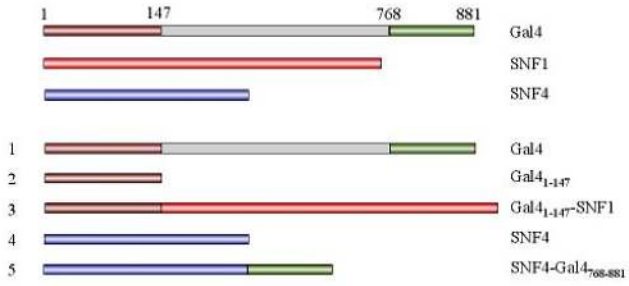


Figure 4.5.6: *Gene constructs with Gal4, SNF1, SNF4*

- These gene constructs were inserted into two different yeast plasmids:

- All constructs containing amino terminal fusions of *GAL4* were put into plasmids containing the *HIS3* gene (coding for a biosynthetic enzyme necessary for the production of the amino acid **histidine**).
- All constructs containing carboxy terminal fusions of *GAL4* were put into plasmids containing the *LEU2* gene (coding for a biosynthetic enzyme necessary for the production of the amino acid **leucine**)

- These plasmids were inserted into a host yeast (GGY1::171)which had the *GAL4* gene deleted, the *LACZ* gene deleted, the *HIS3* and *LEU2* genes mutated to be non-productive and which also contained a *GAL1-LACZ* gene fusion (i.e. a LacZ protein driven from the *GAL1* promoter which has a UAS_{GAL}. This host is thus D*GAL4*, D*LACZ*, *his3*, *leu2*, *gal1-lacZ*.
- Thus, selective pressure for the amino terminal *GAL4* fusions can be maintained by eliminating histidine in the growth media (yeast relies on the *HIS3* gene on the plasmid to make histidine)
- Likewise, selective pressure for the carboxy terminal *GAL4* fusions can be maintained by eliminating leucine in the growth media (yeast relies on the *LEU2* gene on the plasmid to make leucine)
- The host can harbor both kinds of plasmids (in which case the media would lack both histidine and leucine)
- The expression of the fusion constructs on the plasmid is actually regulated by galactose. Adding galactose results in expression of the fusion constructs. However, since the host is D*GAL4* all the other galactose metabolic genes (e.g. *GAL1*) will not be up-regulated in response to added galactose.

The presence of a functional Gal4 protein in the GGY1::171 yeast host will result in the following:

- Expression of the LacZ protein (contained in the host *GAL1-LACZ* gene fusion) will occur. Thus, in the presence of XGAL substrate the yeast colony will turn blue
- **All the host genes which contain an UAS_{GAL} will be up-regulated. This will include all the wild-type genes involved in galactose metabolism (e.g. *GAL1*)**

Experimental Results

Plasmid	LacZ activity
1. None	<1
2. *GAL4*(1-881)	**4,000**
3. *GAL4*(1-147)	<1
4. *GAL4*(1-147)-SNF1	<1
5. SNF4	<1
6. SNF4- *GAL4*(768-881)	<1
7. *GAL4*(1-147)-SNF1; SNF4-*GAL4*(768-881)	**180**
8. *GAL4*(1-147)-SNF1; SNF4	7
9. *GAL4*(1-147); SNF4-*GAL4*(768-881)	<1

- Functional Gal4 protein resulted in expression of the host *GAL1-LACZ* gene
- Co-expression of plasmids containing *GAL4*(1-147)-SNF1; SNF4-*GAL4*(768-881) fusion proteins resulted in expression of the host *GAL1-LACZ* gene, indicating the presence of functional Gal4 protein (i.e. complex formation of the Gal4 fusion proteins)
- The level of LacZ protein was less, however, indicating that the SFN1- and SFN4- Gal4 fusion protein complex was a less efficient as a transcription activator than wild type Gal4 protein
- There was some low level LacZ protein activity with the *GAL4*(1-147)-SNF1; SNF4 protein complex. This suggests that the SNF4 may have some minor affinity for RNA polymerase

---

Conclusions

This method may prove useful for identifying proteins which can form stable complexes (i.e. identifying specific protein:protein interactions). For example, if we want to know which proteins bind to a certain receptor we can make a *GAL4*(1-147)-receptor

fusion protein and screen a cDNA library for potential binding proteins by constructing cDNA-*GAL4*(768-881) fusion constructs. Potential binding proteins from such a library can be screened by looking for blue colonies with XGAL substrate, or selected for by growing the yeast in media with galactose as the carbon source.

This page titled 10.6.4: Protein-Protein Recognition Probed Using a Yeast Transcriptional Activator System is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **4.5: Protein-Protein Recognition Probed Using a Yeast Transcriptional Activator System** by Michael Blaber has no license indicated.

10.6.5: Molecular Imprinting

Mosbach, K. TIBS (1994) 19: 9-14

Molecular recognition is the foundation of many biological processes. A major emphasis in biotechnology is the development of synthetic recognition molecules and systems which are specific for a ligand of interest. One branch of molecular design involves the synthesis of molecules with specific stereochemical structure designed to recognize and interact with a particular ligand. In contrast, molecular imprinting is a method of constructing a molecule, with specific recognition properties, by having the ***ligand itself*** direct the assembly of the desired structure

Technical aspects

The general approach of the technique is to combine the desired "imprint" molecule with a solution of small monomeric molecules which has several desired features. They have a variety of functional groups (e.g. hydrogen bond donors, acceptors, possibly acidic or basic groups, or aliphatic or aromatic groups). One or more of these groups can specifically interact with the imprint molecule (e.g. they can form specific hydrogen bonds). The monomeric molecules can be made to polymerize to form a rigid, or semi-rigid polymer. The imprint molecule is soluble in the monomer solution. The monomers, either in solution or during the polymerization process, do not form covalent bonds with the imprint molecule (the interaction is strictly non-covalent)

After polymerization of the monomers (in the presence of the imprint molecule) the general features of the polymer are as follows:

1. The polymer is ***porous enough*** to allow the imprint molecule to diffuse out (and likewise to diffuse in)
2. The polymer is ***rigid enough*** that the organization of monomeric molecules in response to the imprint molecule is retained.
3. Therefore, the stereochemistry (i.e. size, shape and orientation of appropriate functional groups) of the pocket where the imprint molecules were located is retained.

> *These sites in the polymer constitute an "induced molecular memory" capable of selectively recognizing the imprint molecule*

The typical polymer production technique has been to produce a bulk imprinted polymer and then grind it up to produce small particles (25 mm). Such small beads are appropriate for liquid chromatography methods (i.e. can be used as chromatographic resins and packed in a column). "Suspension" polymerization can yield beads directly from the polymerization process

Most of the reported molecular imprinted polymers reported have been made from monomers of acrylates, styrenes or silicates, and the imprinting step performed in organic solvents. Imprinting in organic solvents (e.g. low dielectric constant solvents) enhances hydrogen bonding (i.e. partial electrostatic) interactions. However, it can limit imprint molecules to those that are soluble in such solvents.

Example 10.6.5.1

Imprinting of the amino acid L-phenylalanine with methacrylic acid monomers and the enantioselective binding of the imprint molecule.

- Step I. "Imprinting"

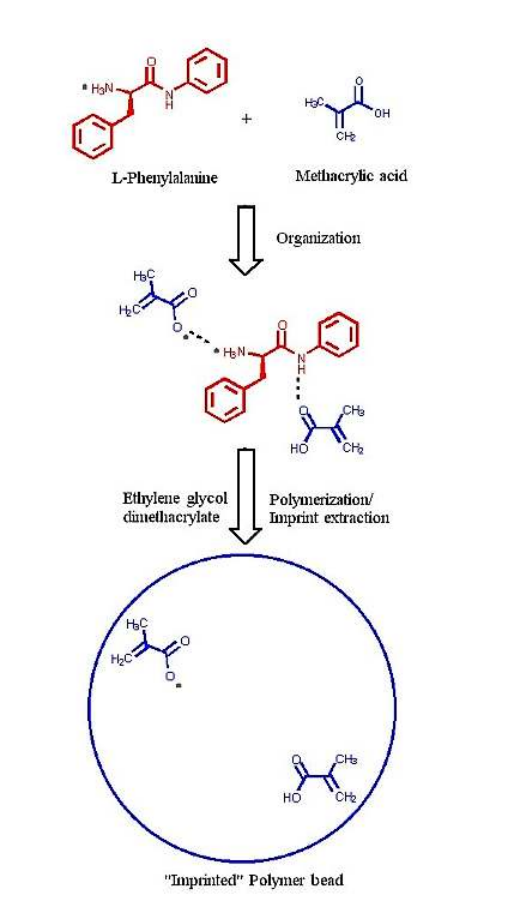


Figure 4.6.1: *Imprinting*

- Step II. Use of the imprinted polymer as a chromatographic resin to preferentially achieve the enantiomeric selection of L-phenylalanine from a racemic mixture of L- and D-phenylalanine

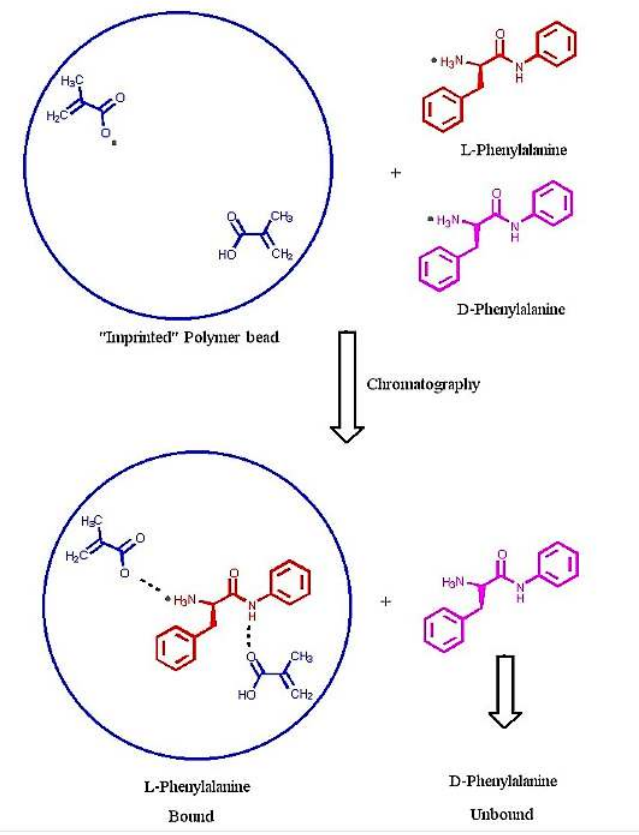


Figure 4.6.2: *Enantiomeric selection*

Applications

Three main areas of application of MIP's include chromatographic matrices for difficult (e.g. enantiomeric) separations, catalytically active polymers or enzyme mimics, and Bio-sensor devices.

Separations

There are about 500 drugs on the market which are optically active, about 90% of these are administered as racemic mixtures. The FDA now requires that for optically active drugs both enantiomers must be treated as separate substances in pharmacokinetic and toxicology profiling. There is a great need for preparative methods of enantiomer separations

Other separations

MIP's made against particular alkaloids or opiates can discriminate between the imprinted compounds and closely related compounds

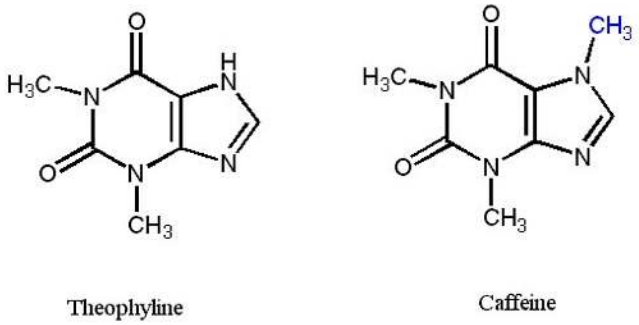


Figure 4.6.3: *Closely related compounds that can be separated with MIP's*

Imprinted polymers as enzyme mimics

If imprints are prepared against *transition state analogs*, the MIP will stabilize the transition state and result in enhanced catalytic rates. Increased catalytic rates (turnover) in comparison to substrate turnover rates in solution have been observed with polymers imprinted with transition state analogs. However, they are generally poor catalysts. This is most likely due to the fact that specific stereochemical placement of catalytic groups was not attempted. Thus, such MIP's represent an appropriate framework within which to place catalytic groups to achieve efficient catalysis

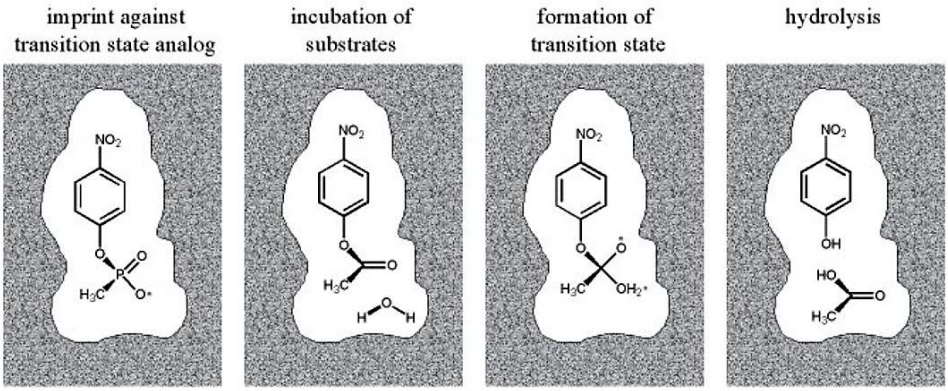


Figure 4.6.4: *Imprints as enhancer for catalytic rates*

Imprinted polymers as substrate selective sensors

"biosensors" are devices which integrate specific receptor molecules with signal transduction devices. The transducer may convert the binding energy of the MIP/analyte into changes in protons, light absorbance, heat, etc. This physical/chemical signal is ampified and output to a measuring device. In principle, the analyte can be any molecule which can be specifically imprinted. For example, an environmental toxin.

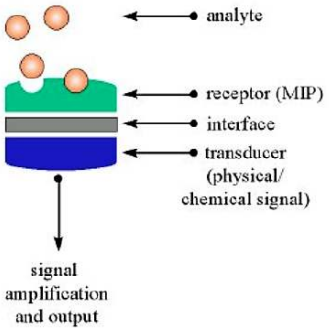


Figure 4.6.5: *Imprinted polymer as biosensor*

Conclusions

Although most work with MIP have involved small molecules, in principle it should be possible to extend MIP utility to include biomolecule recognition (i.e. proteins, nucleic acids). Such MIP should exhibit similar specificities. If MIP can mimic biomolecule binding sites, they may prove useful alternatives for the screening of new inhibitors, or antagonists. One potential limitation is that of "mutagenesis". Can a polymer which has already been imprinted be "mutagenized" (chemically modified?) to produce novel/enhanced recognition characteristics? Another limitation is that the exact stereochemical structure of the imprint is not known. Therefore, the structural information of a useful imprint is not available.

This page titled 10.6.5: Molecular Imprinting is shared under a not declared license and was authored, remixed, and/or curated by Michael Blaber.

- **4.6: Molecular Imprinting** by Michael Blaber has no license indicated.

Index

A

acetyl CoA
7.2.3: Citric Acid Cycle and Oxidative Phosphorylation

activation energy
7.2.1: Energy and Metabolism

active site
7.2.1: Energy and Metabolism

aliphatic hydrocarbon
1.4: Carbon and Functional Groups

allosteric inhibition
7.2.1: Energy and Metabolism

anabolic
7.2.1: Energy and Metabolism

anaerobic cellular respiration
7.2.4: Fermentation

anion
1.2: Atoms, Ions, Molecules

aromatic hydrocarbon
1.4: Carbon and Functional Groups

atom
1.1.1: Properties of life
1.2: Atoms, Ions, Molecules

atomic mass
1.2: Atoms, Ions, Molecules

atomic number
1.2: Atoms, Ions, Molecules

ATP
7.2.2: Glycolysis

ATP synthase
7.2.3: Citric Acid Cycle and Oxidative Phosphorylation

B

balanced chemical equation
1.2: Atoms, Ions, Molecules

biochemistry
1.1.1: Properties of life

bioenergetics
7.2.1: Energy and Metabolism

biosphere
1.1.1: Properties of life

Botany
1.1.1: Properties of life

C

Cardiolipins
5.2: Structure and Function - Lipids and Their Transport
5.2.1: 5.2.1 Optional- other interesting lipids

catabolic
7.2.1: Energy and Metabolism

cation
1.2: Atoms, Ions, Molecules

cell
1.1.1: Properties of life

cell wall
1.1.3: Eukaryotic Cells

central dogma
10.2.4: Central Dogma and the Genetic Code

central vacuole
1.1.3: Eukaryotic Cells

chemical bond
1.2: Atoms, Ions, Molecules

chemical reaction
1.2: Atoms, Ions, Molecules

chemical reactivity
1.2: Atoms, Ions, Molecules

chemiosmosis
7.2.3: Citric Acid Cycle and Oxidative Phosphorylation

Chemiosmotic model
6.4: Electron Transport and Oxidative Phosphorylation

chloroplast
1.1.3: Eukaryotic Cells

chromatography
9.2: Fractionation and Chromatography Techniques

chymotrypsin
3.3: Mechanisms of Catalysis

cilium
1.1.3: Eukaryotic Cells

citric acid cycle
6.3: Citric Acid Cycle and Related Pathways
7.2.3: Citric Acid Cycle and Oxidative Phosphorylation

collagen
2.3: Structure and Function- Proteins II

community
1.1.1: Properties of life

competitive inhibition
7.2.1: Energy and Metabolism

compound
1.2: Atoms, Ions, Molecules

covalent bond
1.2: Atoms, Ions, Molecules

CRISPR
9.9: Genome Editing (CRISPR)

cytoplasm
1.1.3: Eukaryotic Cells

cytoskeleton
1.1.3: Eukaryotic Cells

cytosol
1.1.3: Eukaryotic Cells

D

desmosome
1.1.3: Eukaryotic Cells

DNA (Z Form)
4.2: Structure and Function - Nucleic Acids

E

E. coli
10.5.1: Genetic Transformation (using bacteria and the pGLO plasmid)

ecosystem
1.1.1: Properties of life

electrolyte
1.2: Atoms, Ions, Molecules

electron configuration
1.2: Atoms, Ions, Molecules

electron orbital
1.2: Atoms, Ions, Molecules

electron transfer
1.2: Atoms, Ions, Molecules

electron transport chain
7.2.3: Citric Acid Cycle and Oxidative Phosphorylation

electronegativity
1.2: Atoms, Ions, Molecules

electrophoresis
9.3: Electrophoresis

element
1.2: Atoms, Ions, Molecules

enantiomers
1.4: Carbon and Functional Groups

endergonic
7.2.1: Energy and Metabolism

endocytosis
5.5: Other Considerations in Membranes

endomembrane system
1.1.3: Eukaryotic Cells

endoplasmic reticulum
1.1.3: Eukaryotic Cells

enzyme
7.2.1: Energy and Metabolism

enzyme induction
10.2.5: Gene and Operon

enzyme repression
10.2.5: Gene and Operon

ethidium bromide
10.3.1: Gel Electrophoresis

eukaryote
1.1.1: Properties of life

eukaryotic cell
1.1.2: Prokaryotic Cells and Scale

evolution
1.1.1: Properties of life

exergonic
7.2.1: Energy and Metabolism

Expression Cloning
9.6: Isolating Genes

Expression vectors
9.6: Isolating Genes

extracellular matrix
1.1.3: Eukaryotic Cells

F

feedback inhibition
7.2.1: Energy and Metabolism

fermentation
7.2.4: Fermentation

flagellum
1.1.3: Eukaryotic Cells

Fluorescence Recovery After Photobleaching (FRAP)
9.10: Membrane Dynamics (FRAP)
Fractionation
9.2: Fractionation and Chromatography Techniques
functional group
1.4: Carbon and Functional Groups

G
galactose containing media
10.6.4: Protein-Protein Recognition Probed Using a Yeast Transcriptional Activator System
gap junction
1.1.3: Eukaryotic Cells
gel electrophoresis
10.3.1: Gel Electrophoresis
gene
10.2.5: Gene and Operon
genetic code
10.2.4: Central Dogma and the Genetic Code
geometric isomer
1.4: Carbon and Functional Groups
gluconeogenesis
6.2: Metabolism - Sugars
glycolysis
7.2.2: Glycolysis
glycolytic pathway
6.2: Metabolism - Sugars
Golgi apparatus
1.1.3: Eukaryotic Cells

H
heat energy
7.2.1: Energy and Metabolism
Hind III
9.6: Isolating Genes
homeostasis
1.1.1: Properties of life
hydrocarbon
1.4: Carbon and Functional Groups
hydrogen bond
1.2: Atoms, Ions, Molecules

I
inert gas
1.2: Atoms, Ions, Molecules
introns
10.2.5: Gene and Operon
ionic bond
1.2: Atoms, Ions, Molecules
irreversible chemical reaction
1.2: Atoms, Ions, Molecules
Isoelectric focusing
9.3: Electrophoresis
isomers
1.4: Carbon and Functional Groups
Isoprenoids
6.7: Other Lipids
isotope
1.2: Atoms, Ions, Molecules

K
keratins
2.3: Structure and Function- Proteins II
Kinase
10.1.4: DNA Modifying Enzymes
kinetic energy
7.2.1: Energy and Metabolism

L
Ligases
10.1.4: DNA Modifying Enzymes
Lysis
9.1: Cell Lysis
lysosome
1.1.3: Eukaryotic Cells

M
M13 Phage
10.6.1: M13 Phage
macromolecule
1.1.1: Properties of life
Macropinocytosis
5.5: Other Considerations in Membranes
matter
1.2: Atoms, Ions, Molecules
metabolism
7.2.1: Energy and Metabolism
Methylases
10.1.4: DNA Modifying Enzymes
methylation
10.1.4: DNA Modifying Enzymes
Microarrays
9.4: Detection, identification and quantitation of specific nucleic acids and proteins
microbiology
1.1.1: Properties of life
mitochondria
1.1.3: Eukaryotic Cells
molecular biology
1.1.1: Properties of life
Molecular Imprinting
10.6.5: Molecular Imprinting
molecule
1.1.1: Properties of life
Morpheein model
3.2: Control of Enzymatic Activity

N
neurobiology
1.1.1: Properties of life
neutron
1.2: Atoms, Ions, Molecules
noble gas
1.2: Atoms, Ions, Molecules
Noncompetitive Inhibition
7.2.1: Energy and Metabolism
nonpolar covalent bond
1.2: Atoms, Ions, Molecules
northern blot
9.4: Detection, identification and quantitation of specific nucleic acids and proteins
nuclear envelope
1.1.3: Eukaryotic Cells
Nucleases
10.1.4: DNA Modifying Enzymes
nucleic acids
4.2: Structure and Function - Nucleic Acids
nucleolus
1.1.3: Eukaryotic Cells
nucleus
1.1.3: Eukaryotic Cells

O
operons
10.2.5: Gene and Operon
organ
1.1.1: Properties of life
organ system
1.1.1: Properties of life
organelle
1.1.1: Properties of life
1.1.2: Prokaryotic Cells and Scale
organic molecule
1.4: Carbon and Functional Groups
organism
1.1.1: Properties of life
oxidative phosphorylation
7.2.3: Citric Acid Cycle and Oxidative Phosphorylation

P
paleontology
1.1.1: Properties of life
Palindromes
9.6: Isolating Genes
PCR
10.3.3: Polymerase Chain Reaction (PCR) and Cloning of PCR Products
Perilipin
6.5: Fats and Fatty Acids
periodic table
1.2: Atoms, Ions, Molecules
peroxisome
1.1.3: Eukaryotic Cells
pGLO plasmid
10.5.1: Genetic Transformation (using bacteria and the pGLO plasmid)
Phage Display Libraries
10.6.2: M13 Phage Display Libraries
phagocytosis
5.5: Other Considerations in Membranes
Phosphatase
10.1.4: DNA Modifying Enzymes
Photophosphorylation
6.8: Energy - Photophosphorylation
phylogenetic tree
1.1.1: Properties of life
plasma membrane
1.1.3: Eukaryotic Cells
plasmodesma
1.1.3: Eukaryotic Cells

Polyacrylamide gel electrophoresis
9.3: Electrophoresis
polymerase chain reaction (PCR)
9.7: Polymerase Chain Reaction (PCR)
10.5.5: Polymerase Chain Reaction (PCR)
Polymerases
10.1.4: DNA Modifying Enzymes
population
1.1.1: Properties of life
Porphyria
6.7: Other Lipids
potential energy
7.2.1: Energy and Metabolism
product
1.2: Atoms, Ions, Molecules
prokaryote
1.1.1: Properties of life
prokaryotic cell
1.1.2: Prokaryotic Cells and Scale
promoter
4.6: Transcription
proton
1.2: Atoms, Ions, Molecules

R
radioisotope
1.2: Atoms, Ions, Molecules
Replication
4.3: DNA Replication
Restriction Enzymes
9.6: Isolating Genes
Restriction Mapping
10.5.4: Restriction Mapping
ribosome
1.1.3: Eukaryotic Cells
rough endoplasmic reticulum
1.1.3: Eukaryotic Cells

S
secondary structure
2.3: Structure and Function- Proteins II
SELEX
10.6.3: SELEX (Selective Evolution of Ligands by Exponential Enrichment)
serine proteases
3.3: Mechanisms of Catalysis
signaling
7.1: Signaling
size exclusion chromatography
9.2: Fractionation and Chromatography Techniques
smooth endoplasmic reticulum
1.1.3: Eukaryotic Cells
SNARE proteins
5.5: Other Considerations in Membranes
Southern blot
9.4: Detection, identification and quantitation of specific nucleic acids and proteins
sphingolipids
6.7: Other Lipids
squalene
6.7: Other Lipids
statins
6.7: Other Lipids
structural isomers
1.4: Carbon and Functional Groups
substituted hydrocarbon
1.4: Carbon and Functional Groups
substrate
7.2.1: Energy and Metabolism
subtilisin
3.3: Mechanisms of Catalysis

T
tertiary structure
2.3: Structure and Function- Proteins II
thermodynamics
7.2.1: Energy and Metabolism
tight junction
1.1.3: Eukaryotic Cells
Transcription
4.6: Transcription
transcriptional activator protein
10.6.4: Protein-Protein Recognition Probed Using a Yeast Transcriptional Activator System
transcriptome
9.5: Transcriptomics
transformation
10.5.1: Genetic Transformation (using bacteria and the pGLO plasmid)
translation
4.8: Translation
10.2.8: mRNA Translation

U
Upstream Activator Sequences (UAS's)
10.6.4: Protein-Protein Recognition Probed Using a Yeast Transcriptional Activator System
urea cycle
6.6: Amino Acids and the Urea Cycle

V
vacuole
1.1.3: Eukaryotic Cells
van der Waals interaction
1.2: Atoms, Ions, Molecules
Vesicle
1.1.3: Eukaryotic Cells
viruses
8.7.1: Viruses

W
Western blot
9.4: Detection, identification and quantitation of specific nucleic acids and proteins

Z
zoology
1.1.1: Properties of life

Glossary

Sample Word 1 | Sample Definition 1

Detailed Licensing

Overview

Title: Survey of Biochemistry and Biotechnology

Webpages: 136

Applicable Restrictions: Noncommercial

All licenses found:

- Undeclared: 45.6% (62 pages)
- CC BY-NC-SA 4.0: 39.7% (54 pages)
- CC BY 4.0: 14.7% (20 pages)

By Page

- Survey of Biochemistry and Biotechnology - *Undeclared*
  - Front Matter - *Undeclared*
    - TitlePage - *Undeclared*
    - InfoPage - *Undeclared*
    - Table of Contents - *Undeclared*
    - Licensing - *Undeclared*
  - 1: Chemical and Biological Foundations - *Undeclared*
    - 1.1: Biological Foundations - *CC BY-NC-SA 4.0*
      - 1.1.1: Properties of life - *CC BY 4.0*
      - 1.1.2: Prokaryotic Cells and Scale - *CC BY 4.0*
      - 1.1.3: Eukaryotic Cells - *CC BY 4.0*
    - 1.2: Atoms, Ions, Molecules - *CC BY 4.0*
    - 1.3: Intermolecular Forces - *CC BY 4.0*
    - 1.4: Carbon and Functional Groups - *CC BY 4.0*
    - 1.5: Water, Equilibrium, and Buffers - *CC BY-NC-SA 4.0*
  - 2: Structure and Function- Proteins - *Undeclared*
    - 2.1: Structure and Function - Amino Acids - *CC BY-NC-SA 4.0*
    - 2.2: Structure and Function- Proteins I - *CC BY-NC-SA 4.0*
    - 2.3: Structure and Function- Proteins II - *CC BY-NC-SA 4.0*
  - 3: Catalysis - *Undeclared*
    - 3.1: Basic Principles of Catalysis - *CC BY-NC-SA 4.0*
    - 3.2: Control of Enzymatic Activity - *CC BY-NC-SA 4.0*
    - 3.3: Mechanisms of Catalysis - *CC BY-NC-SA 4.0*
  - 4: Structure and Function- Nucleic Acids - *Undeclared*
    - 4.1: Prelude to Information Processing - *CC BY-NC-SA 4.0*
    - 4.2: Structure and Function - Nucleic Acids - *CC BY-NC-SA 4.0*
    - 4.3: DNA Replication - *CC BY-NC-SA 4.0*
    - 4.4: DNA Repair - *CC BY-NC-SA 4.0*
    - 4.5: Genes and Genomes - *CC BY-NC-SA 4.0*
    - 4.6: Transcription - *CC BY-NC-SA 4.0*
      - 4.6.1: RNA Processing - *CC BY-NC-SA 4.0*
    - 4.7: RNA Processing - *CC BY-NC-SA 4.0*
    - 4.8: Translation - *CC BY-NC-SA 4.0*
    - 4.9: Gene Expression - *CC BY-NC-SA 4.0*
  - 5: Structure and Function- Carbohydrates and Lipids - *Undeclared*
    - 5.1: Structure and Function- Carbohydrates - *CC BY-NC-SA 4.0*
    - 5.2: Structure and Function - Lipids and Their Transport - *CC BY-NC-SA 4.0*
      - 5.2.1: 5.2.1 Optional- other interesting lipids - *CC BY-NC-SA 4.0*
        - 5.2.1.1: Bonus- other interesting lipids - *Undeclared*
    - 5.3: Basic Concepts in Membranes - *CC BY-NC-SA 4.0*
    - 5.4: Transport Across Membranes - *CC BY-NC-SA 4.0*
      - 5.4.1: Optional deep dive in to membrane transport - *CC BY-NC-SA 4.0*
    - 5.5: Other Considerations in Membranes - *CC BY-NC-SA 4.0*
  - 6: Energy and Metabolism - *Undeclared*
    - 6.1: Basics of Energy - *CC BY-NC-SA 4.0*
    - 6.2: Metabolism - Sugars - *CC BY-NC-SA 4.0*
    - 6.3: Citric Acid Cycle and Related Pathways - *CC BY-NC-SA 4.0*
    - 6.4: Electron Transport and Oxidative Phosphorylation - *CC BY-NC-SA 4.0*
    - 6.5: Fats and Fatty Acids - *CC BY-NC-SA 4.0*

- 6.6: Amino Acids and the Urea Cycle - *CC BY-NC-SA 4.0*
- 6.7: Other Lipids - *CC BY-NC-SA 4.0*
- 6.8: Energy - Photophosphorylation - *CC BY-NC-SA 4.0*

7: Regulation of Metabolism and Homeostasis - *CC BY-NC-SA 4.0*

- 7.1: Signaling - *CC BY-NC-SA 4.0*
- 7.2: How Cells Obtain Energy - *CC BY 4.0*
  - 7.2.1: Energy and Metabolism - *CC BY 4.0*
  - 7.2.2: Glycolysis - *CC BY 4.0*
  - 7.2.3: Citric Acid Cycle and Oxidative Phosphorylation - *CC BY 4.0*
  - 7.2.4: Fermentation - *CC BY 4.0*
  - 7.2.5: Connections to Other Metabolic Pathways - *CC BY 4.0*
  - 7.2.E: How Cells Obtain Energy (Exercises) - *CC BY 4.0*
- 7.3: Section - place holder - *CC BY-NC-SA 4.0*

8: Biotechnology and Other Applications of Biochemistry - *CC BY-NC-SA 4.0*

- 8.1: Section 1- - *CC BY-NC-SA 4.0*
- 8.2: Section 2- - *CC BY-NC-SA 4.0*
- 8.3: Section 3- - *CC BY-NC-SA 4.0*
- 8.4: Section 4- - *CC BY-NC-SA 4.0*
- 8.5: Section 5- - *CC BY-NC-SA 4.0*
- 8.6: Section 6- - *CC BY-NC-SA 4.0*
- 8.7: The Immune System and Disease - *CC BY 4.0*
  - 8.7.1: Viruses - *CC BY 4.0*
  - 8.7.2: Innate Immunity - *CC BY 4.0*
  - 8.7.3: Adaptive Immunity - *CC BY 4.0*
  - 8.7.4: Disruptions in the Immune System - *CC BY 4.0*
  - 8.7.E: The Immune System and Desease (Exercises) - *CC BY 4.0*
- 8.8: Biotechnology - *CC BY 4.0*

9: Basic Techniques - *CC BY-NC-SA 4.0*

- 9.1: Cell Lysis - *CC BY-NC-SA 4.0*
- 9.2: Fractionation and Chromatography Techniques - *CC BY-NC-SA 4.0*
- 9.3: Electrophoresis - *CC BY-NC-SA 4.0*
- 9.4: Detection, identification and quantitation of specific nucleic acids and proteins - *CC BY-NC-SA 4.0*
- 9.5: Transcriptomics - *CC BY-NC-SA 4.0*
- 9.6: Isolating Genes - *CC BY-NC-SA 4.0*
- 9.7: Polymerase Chain Reaction (PCR) - *CC BY-NC-SA 4.0*
- 9.8: Reverse Transcription - *CC BY-NC-SA 4.0*
- 9.9: Genome Editing (CRISPR) - *CC BY-NC-SA 4.0*
- 9.10: Membrane Dynamics (FRAP) - *CC BY-NC-SA 4.0*

10: Supplemental Modules (Biochemistry) - *Undeclared*

- 10.1: DNA - *Undeclared*
  - 10.1.1: DNA as Genetic Material - *Undeclared*
  - 10.1.2: Structure of DNA and RNA - *Undeclared*
  - 10.1.3: Bacterial Restriction/Modification system - *Undeclared*
  - 10.1.4: DNA Modifying Enzymes - *Undeclared*
  - 10.1.5: DNA Replication - Introduction to Prokaryotic replication - *Undeclared*
  - 10.1.6: DNA Supercoiling and Topoisomerases - *Undeclared*
- 10.2: Bacteria - *Undeclared*
  - 10.2.1: The Bacteria - An Introduction - *Undeclared*
  - 10.2.2: Bacterial Growth and Reproduction - *Undeclared*
  - 10.2.3: Extrachromosomal elements, plasmids, selectable markers - *Undeclared*
  - 10.2.4: Central Dogma and the Genetic Code - *Undeclared*
  - 10.2.5: Gene and Operon - *Undeclared*
  - 10.2.6: The lac Operon; CAP site; DNA footprinting - *Undeclared*
  - 10.2.7: Transcriptional Regulation- Transcription termination, the trp operon - *Undeclared*
  - 10.2.8: mRNA Translation - *Undeclared*
- 10.3: 3. Biotechnology 1 - *Undeclared*
  - 10.3.1: Gel Electrophoresis - *Undeclared*
  - 10.3.2: DNA Sequence Analysis - *Undeclared*
  - 10.3.3: Polymerase Chain Reaction (PCR) and Cloning of PCR Products - *Undeclared*
  - 10.3.4: Prokaryotic Expression Vectors - *Undeclared*
  - 10.3.5: Protein Sequencing, Peptide Mapping, Synthetic Genes - *Undeclared*
  - 10.3.6: cDNA and Genomic Libraries - *Undeclared*
  - 10.3.7: Protein Purification - *Undeclared*
- 10.4: 5. Lab Notes Part 1 - *Undeclared*
  - 10.4.1: Basic practices and techniques, Laboratory notebooks, Presentation of data, Sections of a scientific report - *Undeclared*
  - 10.4.2: Spectrophotometry - *Undeclared*

- 10.4.3: Quantification of Protein Concentration - *Undeclared*
- 10.4.4: Chromatography - *Undeclared*
- 10.4.5: Gel Electrophoresis of Proteins - *Undeclared*
- 10.4.6: Introduction to Protein Purification - *Undeclared*

10.5: 6. Lab Notes Part 2 - *Undeclared*

- 10.5.1: Genetic Transformation (using bacteria and the pGLO plasmid) - *Undeclared*
- 10.5.2: Enzyme kinetics - *Undeclared*
- 10.5.3: Ligand binding - *Undeclared*
- 10.5.4: Restriction Mapping - *Undeclared*
- 10.5.5: Polymerase Chain Reaction (PCR) - *Undeclared*
- 10.5.6: Use of PC and internet for biochemical research - *Undeclared*

10.6: 4. Biotechnology 2 - *Undeclared*

- 10.6.1: M13 Phage - *Undeclared*
- 10.6.2: M13 Phage Display Libraries - *Undeclared*
- 10.6.3: SELEX (Selective Evolution of Ligands by Exponential Enrichment) - *Undeclared*
- 10.6.4: Protein-Protein Recognition Probed Using a Yeast Transcriptional Activator System - *Undeclared*
- 10.6.5: Molecular Imprinting - *Undeclared*

Back Matter - *Undeclared*

- Index - *Undeclared*
- Glossary - *Undeclared*
- Detailed Licensing - *Undeclared*