FUNDAMENTALS OF BIOCHEMISTRY III

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SECTION OVERVIEW

Fundamentals of Biochemistry (Jakubowski and Flatt)

With contributions from William (Bill) W. Christie, Kristen Procko, and Logan Hallee; Editing contributions from Ellen Anderson and Brianna Bibel

This text is designed for the two-semester introductory biochemistry course but could be customized for a one-semester course as well. Biochemistry is the application of chemistry to the study of biological processes at the cellular and molecular levels. It emerged as a distinct discipline around the beginning of the 20th century when scientists combined chemistry, physiology, and biology to investigate the chemistry of living systems. Biochemistry is both a life science and a chemical science - it explores the chemistry of living organisms and the molecular basis for the changes occurring in living cells. It uses the methods of chemistry, physics, molecular biology, and immunology to study the structure and behavior of the complex molecules found in biological material and the ways these molecules interact to form cells, tissues, and whole organisms. The chapter organization generally follows the traditionally used biochemistry texts to allow easier adoption by faculty and use by students.

New! Each chapter section now has learning goals and end-of-chapter summaries designed for Junior and Senior Biochemistry students/majors (3/6/25)

Important! Chapter32 - Biochemistry and Climate Change (1/14/23)

Front Matters (Preface, Learning Goals, Author Info, etc)

Fundamentals of Biochemistry Vol. I - Structure and Catalysis

Fundamentals of Biochemistry Vol. II - Bioenergetics and Metabolism

Fundamentals of Biochemistry Vol. III - Information Pathways

Fundamentals of Biochemistry Vol. IV - Signaling, Integration of Metabolism, and Special Topics

Fundamentals of Biochemistry Vol. V - Literature-based Guided Assessments and iCn3D Molecular Modeling Tutorials

CHAPTER OVERVIEW

1: The Foundations of Biochemistry

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1.1: Cellular Foundations

Learning Goals (ChatGPT o3-mini)

Below is a list of learning goals tailored for junior and senior biochemistry majors based on the provided information:

1. **Differentiate Cell Types and Organelles**
   - Explain the fundamental differences between prokaryotic and eukaryotic cells.
   - Identify and describe the roles of key organelles in a eukaryotic cell, with emphasis on how compartmentalization supports cellular functions.
2. **Understand Biomolecular Diversity and Distribution**
   - List and characterize the four major classes of biomolecules (lipids, proteins, nucleic acids, and carbohydrates).
   - Trace the "history" of these biomolecules—from synthesis, modification, to degradation—and explain their spatial distribution within the cell.
3. **Analyze Metabolic Pathways and Energy Transformations**
   - Define metabolism in terms of energy balance, distinguishing between exergonic (energy-releasing) and endergonic (energy-consuming) reactions.
   - Differentiate between catabolic pathways (breakdown of compounds) and anabolic pathways (synthesis of new compounds), including the energy implications of each.
4. **Examine Enzyme Catalysis and Regulation**
   - Describe the role of enzymes as catalysts in metabolic reactions, including how they lower activation energies and speed up chemical reactions.
   - Compare and contrast the lock and key model with the induced fit model of enzyme-substrate binding.
   - Explain how enzyme activity can be regulated through factors such as allosteric modulation, inhibitors, and environmental changes.
5. **Investigate Cellular Transport Mechanisms**
   - Outline the mechanisms of molecular transport across cellular membranes, including passive diffusion, facilitated diffusion, and active transport.
   - Discuss the roles of membrane proteins such as transporters, ion channels, pores, and carriers in regulating the intracellular environment.
6. **Explore Cellular Organization and Architecture**
   - Describe the structure and function of the cytoskeleton and its role in maintaining cell shape, facilitating intracellular transport, and supporting dynamic changes during processes like cell migration.
   - Explain how the crowded intracellular environment influences biomolecular interactions, protein stability, and reaction kinetics.
7. **Understand Phase Separation and Cellular Substructures**
   - Illustrate how phase transitions (e.g., liquid/liquid demixing) contribute to the formation of cellular substructures such as lipid droplets, membrane rafts, and ribonucleoprotein complexes.
   - Analyze the role of intermolecular forces (ionic interactions, dipole-dipole, ion-dipole, and London dispersion forces) in driving the assembly and function of these structures.
8. **Integrate Chemical Principles with Biological Function**
   - Connect chemical concepts such as reaction kinetics, thermodynamics, and intermolecular forces to biological processes like signal transduction, metabolism, and cellular homeostasis.
   - Apply this integrated understanding to explain how changes at the molecular level can affect cell function and contribute to disease states or pharmacological outcomes.

These learning goals aim to ensure that students not only grasp the biochemical principles underpinning cell structure and function but also appreciate how these concepts integrate into the broader context of cellular physiology and pathology.

Introduction

You have probably studied the cell many times, either in high school or in college biology classes. Many websites review prokaryotic (bacterial and archaeal) and eukaryotic cells (including protists, fungi, plants, and animals). All cells share similar structural components, including genetic material in the form of chromosomes, a membrane-bound lipid bilayer that separates the inside from the outside of the cell, and ribosomes responsible for protein synthesis. This tutorial is designed specifically from the viewpoint of chemistry. It explores four classes of biomolecules that are also present in all cell types (lipids, proteins, nucleic acids, and carbohydrates) and describes, in a simplified pictorial manner, where they are found, produced, and degraded in a typical eukaryotic animal cell (i.e., their lifecycle). This cell review focuses on the organelle structures common in eukaryotic cells. Subsequent chapters will concentrate on the structure and function of specific biomolecules.

Let's think of a cell as a chemical factory that designs, imports, synthesizes, uses, exports, and degrades various chemicals (in the case of the cell, these include lipids, proteins, nucleic acids, and carbohydrates). It must also determine or sense the amount of raw and finished chemicals available and respond to its own and external needs by adjusting production accordingly. ***Biochemistry*** is the branch of science that studies the chemical processes within a cell. Understanding these processes can also lend insight into disease states and the pharmacological effects of toxins, drugs, and other medicines within the body.

The building and breaking down of life-sustaining chemicals within an organism is known as ***Metabolism.*** Overall, the metabolism involves: (1) the net exergonic, energy-releasing metabolism of food/fuel food to power endergonic, energy-requiring cellular processes; (2) the conversion of food/fuel to building blocks for the synthesis of proteins, lipids, nucleic acids, and other biomolecules and (3) the elimination of waste products. These processes enable organisms to grow, reproduce, maintain their structures, and adapt to their environments.

Metabolic reactions may be categorized as ***catabolic***– the *breaking down* of compounds (for example, the breaking down of proteins into amino acids during digestion); or ***anabolic*** – the *building up* (synthesis) of compounds (such as proteins, carbohydrates, lipids, and nucleic acids). Typically, catabolism releases energy, while anabolism consumes it.

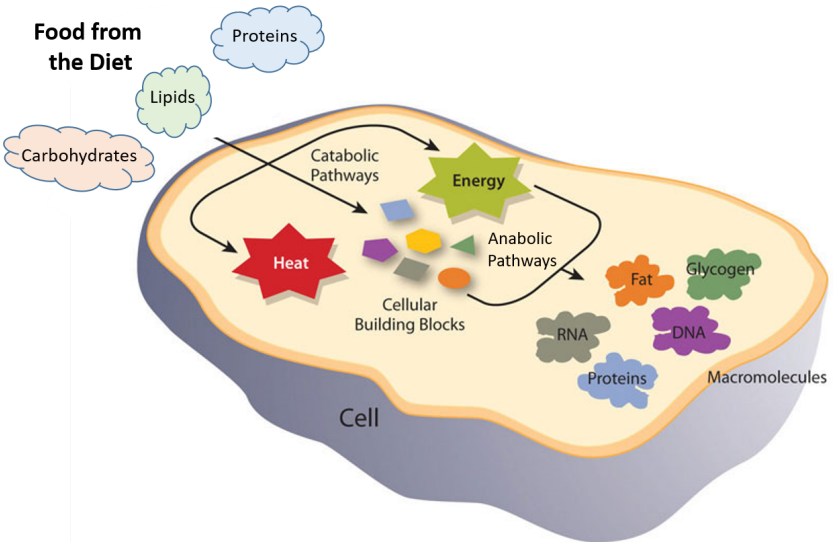


Figure 1.1 Catabolic and Anabolic Reactions. Catabolic reactions break molecules into smaller components, whereas anabolic reactions build larger molecules from smaller molecules. Catabolic reactions usually release energy, whereas anabolic processes usually require energy. (CC BY-SA-NC 3.0; anonymous)

The chemical reactions of metabolism are organized into metabolic pathways, in which one chemical is transformed through a series of steps into another chemical, each step often facilitated by a specific ***enzyme***. ***Enzymes*** are crucial to metabolism because they act as catalysts, allowing reactions to proceed more rapidly. Additionally, enzymes can provide a mechanism for cells to regulate the rate of a metabolic reaction in response to changes in the cell's environment or to signals from other cells, through the activation or inhibition of the enzyme's activity. Enzymes can also enable organisms to drive desirable reactions that require energy, which would not occur spontaneously, by coupling them to spontaneous reactions that release energy. Enzyme shape is critical to the enzyme's function as it determines the specific binding of a reactant. This can occur by a ***lock and key model,*** where the reactant is the exact shape of the enzyme binding site, or by an ***induced fit model***, where the contact of the reactant with the

protein causes the shape of the protein to change to bind to the reactant. The catalytic mechanisms, kinetics, and regulatory pathways of enzymes will be studied in detail within this text.

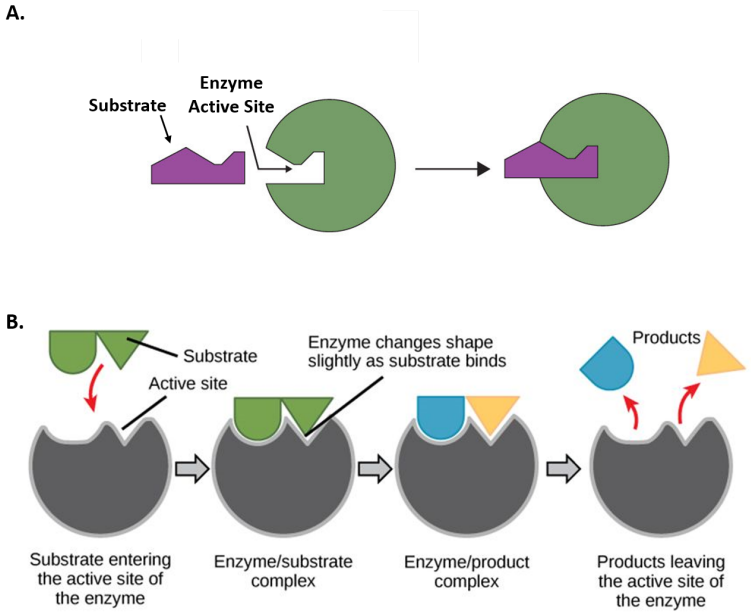


Figure 1.2 Mechanisms of Enzyme-Substrate Binding. (A) In the Lock and Key Model, substrates fit into the enzyme's active site without further modifications to the enzyme's shape. (B) In the Induced Fit Model, substrate interaction causes the enzyme's shape to change to better fit the substrate and mediate the chemical reaction. Figure 1.2A was modified from Socratic, and Figure 1.2B was modified from Concepts in Biology

Within eukaryotic cells, the metabolic machinery allows for the construction of membrane-bound ***organelle*** structures that help compartmentalize cellular functions. Therefore, ***organelles, having discrete cellular functions, can be considered 'little organs' within the cell***. The figure of the cell below and in the other linked sites was made available with the kind permission of Liliana Torres. For more detailed information, click on the blue hyperlinks for some organelles.

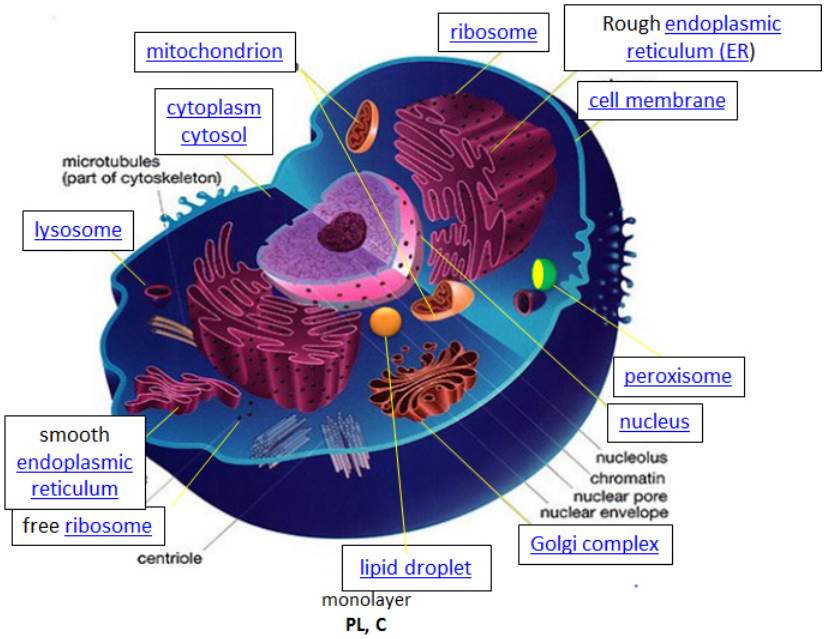


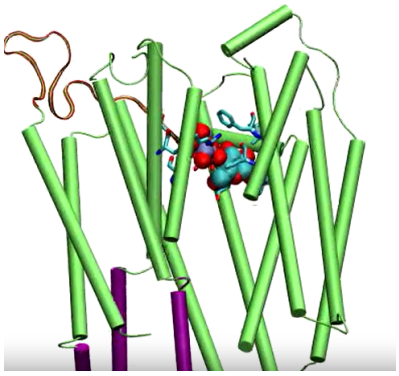
Figure 1.3 Structure of a Typical Eukaryotic Animal Cell. The original figure was acquired from Liliana Torres at: http://torresbioclan.pbworks.com/w/page/22377234/Spikefish%20About%20Cells Use with permission from Liliana Torres. Also at www.animalport.com/animal-cells.html

Design – The design for a cell primarily resides in the blueprint for the cell, the genetic code, which comprises the DNA in the cell nucleus and a small amount in the mitochondria. The DNA blueprint must be read out (transcribed) by DNA readers (DNA-

dependent RNA polymerases) to form RNA. Since DNA and RNA are nucleic acids composed of deoxynucleotides (for DNA) and nucleotides (for RNA), this process is called transcription. A type of RNA, messenger RNA, is then decoded to form a new kind of polymer, a protein, composed of amino acid monomers. This process is called translation (analogous to converting an English sentence into a Spanish one). In a nanomachine, the ribosome, which contains RNA and protein subunits, interacts with the messenger RNA and incoming transfer RNA, each connected to individual amino acids, to create a protein. The genetic code has a master plan that determines the sequence of all cellular proteins, catalyzing nearly all cellular activities, including catalysis, motility, and architectural structure. In contrast to DNA, RNA, and protein polymers, the length and sequence of polysaccharide polymers and lipids are not determined by a template, but rather by the enzymes that catalyze their synthesis.

Import/Export: Many of the chemical constituents of the cell arise not from direct synthesis but from the import of both small and large molecules. The imported molecules must pass through the cell membrane and, in some cases, through additional membranes if they need to reside inside membrane-bound organelles. Molecules can move into the cell by passive diffusion across the membrane, but their movement is usually “facilitated” by a membrane transporter protein. Molecules can also move against a concentration gradient in a process called “active transport.” Given the amphiphilic nature of the bilayer (polar head group exterior, nonpolar interior), you would expect that polar molecules like glucose would have difficulty moving across the membrane by passive diffusion. Typically, only small nonpolar molecules move across the membrane via passive transport. Membrane-bound transport proteins are involved in the movement of both nonpolar and polar molecules.

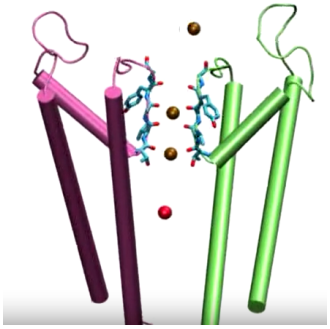
- ***transporters, carrier proteins, and permeases*:** These membrane proteins facilitate the movement of specific ligand molecules across a membrane, typically down a concentration gradient. Click the image below for a computer (molecular dynamics) simulation of the facilitated diffusion of lactose across the membrane by a membrane protein (lactose permease).



Morten Ø. Jensen et al. Sugar Transport across Lactose Permease Probed by Steered Molecular Dynamics. Open Archive. DOI:https://doi.org/10.1529/biophysj.107.103994. Theoretical and Computational Biophysics Group, Beckman Institute, Urbana, Illinois USA. Department of Physics, University of Illinois at Urbana-Champaign, Urbana, Illinois USA Creative Commons licensing

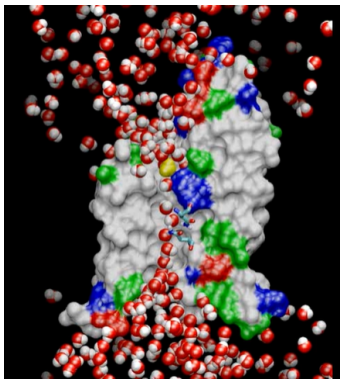
https://www.cell.com/cms/10.1529/bio...329ed/mmc2.mp4

- ***ion channels:*** These membrane proteins allow the flow of ions across membranes. Some are permanently open (nongated), while others are gated, opening or closing depending on the presence of ligands that bind to the protein channel and the local environment of the protein in the membrane. The flow of ions through the channel proceeds in a thermodynamically favored direction, which depends on their concentration and voltage gradients across the membrane.



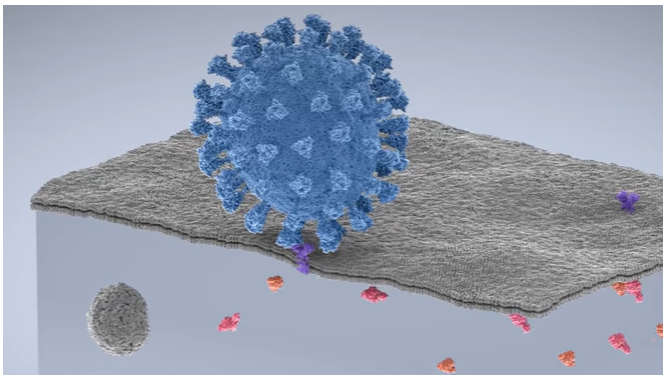
Fatemeh Khalili-Araghi et al. Dynamics of K^+ Ion Conduction through Kv1.2. Open ArchiveDOI:https://doi.org/10.1529/biophysj.106.091926. Theoretical and Computational Biophysics Group, Beckman Institute, Urbana, Illinois USA. Department of Physics, University of Illinois at Urbana-Champaign, Urbana, Illinois

- ***Pores*:** Some membranes (nuclear, mitochondria) assemble proteins (such as porins) to form large but regulated pores. Porins are found in mitochondrial membranes, while nucleoporins are found in the nuclear membrane. Small molecules can generally pass through these membrane pores, while large ones are selected based on their tendency to form transient intermolecular attractive forces with the proteins in the pore. The following link shows the diffusion of water through aquaporin. animation of water diffusion through the aquaporin channel,



Water Transport in Aquaporins. https://www.ks.uiuc.edu/Research/aquaporins/. Theoretical and Computational Biophysics Group, Beckman Institute, Urbana, Illinois USA. Department of Physics, University of Illinois at Urbana-Champaign, Urbana, Illinois

- ***Endocytosis*:** Very large particles [for example, Low-Density Lipoproteins (LDL) and viruses] can enter a cell through **endocytosis**. Initially, the LDL or virus binds to a receptor on the cell's surface. This triggers a series of events that leads to the invagination of the cell membrane at that point. This eventually pinches off to form an ***endosomal vesicle*** surrounded by clathrin, a protein. “Early” endosomes can pick up new proteins and other constituents and shed them as they move and mature through the cell. During this maturation process, protein pumps in the endosome lead to a decrease in the endosomal pH, which can result in conformational changes in protein structure and protein shedding. Eventually, the “late” endosome reaches and fuses with the lysosome, an internal organelle that contains enzymes for degradation. Undegraded components, such as viral nucleic acids or cholesterol, are delivered to the cell. This transport can also go in the reverse direction (called ***exocytosis***) and recycle receptors to the cell membrane. Likewise, vesicles pinched off from the Golgi complex can fuse with endosomes, with some components surviving the process to reenter the Golgi.



Alexey Solodovnikov (Idea, Producer, CG, Editor), Valeria Arkhipova (Scientific Consultant). Endocytosis. For example, coronavirus binds to the ACE2 receptor of the epithelial cell. CC BY-SA 4.0 <https://creativecommons.org/licenses/by-sa/4.0>, via Wikimedia Commons

Synthesize/Degrade: Cells must synthesize and degrade small molecules, as well as larger polymeric proteins, carbohydrates, lipids, and nucleic acids. The ***anabolic*** (synthetic) and ***catabolic*** (degradative) pathways are often compartmentalized in time and space within a cell. For example, fatty acid synthesis takes place in the cytoplasm, whereas fatty acid oxidation occurs in the

mitochondria. Proteins are synthesized in the cytoplasm or completed in the endoplasmic reticulum (for membrane and exported proteins). They are degraded in the lysosome or, more importantly, in a large multimolecular structure in the cell called the proteasome.

Key Characteristics of a Cell

Let's consider some key cell characteristics as a prelude for later chapters.

Cells and their internal compartments have regulated concentrations of ions and hydronium ions.

As expected the pH of the ***cytosol*** (the aqueous substance surrounding all the organelles within the cell) varies from about 7.0-7.4, depending on the metabolic state of the cell. Some organelles have proton transporters that can significantly alter the pH inside an organelle. For example, the pH inside the lysosome, a degradative organelle, is about 4.8. Furthermore, creating a pH gradient across the inner mitochondrial membrane is sufficient to drive the thermodynamically unfavored synthesis of ATP.

Compared to the extracellular fluid, the concentration of potassium ions is higher inside the cell. In contrast, sodium, chloride, and calcium ion concentrations are higher outside the cell (see table below). Ion transporters and channels maintain these concentration gradients and require energy expenditure, ultimately in the form of ATP hydrolysis, to do so. Changes in these concentrations are integral to the signaling system used by the cell to sense and respond to changes in its external and internal environments. The table below shows approximate ion concentrations in the cell.

Table 1.1 Average Cellular and Extracellular Ion Concentrations

Ion	Inside (mM)	Outside (mM)
Na^+	140	5
K^+	12	140
Cl-	4	15
Ca^{2+}	1 uM	2

Cells have an internal framework that provides architectural and internal structural support

The "cytoskeletal" architecture of a cell (with molecular "cables"- and "girder-like" structures) is superficially similar to that of a factory. The cell's internal framework, the ***cytoskeleton,*** is composed of microfilaments, intermediate filaments, and microtubules. These, in turn, are built from proteins that self-assemble to form the internal architecture. Parts of the cytoskeleton can be seen in Figure 1.4.

Figure 1.4 Cellular Architecture. The architecture and organization of structural components within a cell *(right picture)* are analogous to the organization seen within a warehouse *(left picture).* In the right image, bovine pulmonary artery endothelial cells have been stained to indicate the nucleus (blue color), tubulin cytoskeletal proteins (fluorescent green color), and F-actin cytoskeletal proteins (fluorescent red color). Source of the factory picture: http://www.cybercom.net/~copters/trips/pictures/factory_inside.jpg Source of the fluorescent cell picture: http://en.Wikipedia.org/wiki/File:FluorescentCells.jpg

Microfilaments of actin monomers (stained with a red/orange fluorophore) and microtubules, which offer more structural support made of tubulin monomers (stained green) and the blue-stained nucleus, are shown in the image. Organelles are supported and organized by the cytoskeleton (primarily microtubules). Actin (stained orange) and spectrin microfilaments support the cell membrane underneath the inner leaflet. Motor proteins like myosin (that moves along actin microfilaments) and dynein and kinesin

(that move along tubulin microtubules) carry cargo (vesicles, organelles) directionally. The cell is not a disorganized collection of molecules and organelles. Instead, it is highly organized to optimize chemical production, use, and degradation.

Cells have various shapes. Some circulating immune cells must slip through the cells that line capillary walls to migrate to sites of infection. The same process occurs when tumor cells metastasize and escape to other sites in the body. To do so, the cell must drastically change shape, a response that requires the dissociation of the cytoskeleton polymers into monomers, which are later available for polymerization. The following video shows the mobility and flexibility of a Killer T-cell as it attacks and kills a cancerous cell.

Video 1.1 Killer T Cell Attacking Cancer. Video available on YouTube through Creative Commons by Cambridge University

The cell is an amazingly crowded place

In chemistry labs, we typically work with dilute solutions of solute molecules in a solvent. You have probably heard that the body is composed of 68% water, but the water concentration depends on the cellular environment. Solute molecules like protein and carbohydrates are densely packed. Cells are so crowded that the space between larger molecules like proteins is typically smaller than that of a single protein. Studies have shown that the stability of a protein is increased in such conditions, which would help keep the protein in the correctly folded, native state. Another consequence of high intracellular concentrations is that it limits the diffusion of molecules throughout the cell, as expected from an equilibrium perspective in dilute solutions. Thus, cytoplasmic cellular functions can be highly localized within specific cell regions, creating unique microenvironments and higher differentiation potential within a single cell.

Hence, the study of biomolecules in dilute solutions in the lab may not reveal the actual complexities of interactions and activities of the same molecule *in vivo*. Recently, investigators have added a neutral copolymer of sucrose and epichlorohydrin to cells *in vitro*. These particles induced the organization of extracellular molecules secreted by the cell, forming an organized extracellular "matrix," which induced the organization of the microfilaments on the inside and induced changes in cell activity.[1] Furthermore, *in vitro* enzyme activity of a key enzyme in glycolysis dramatically increases under crowded conditions.[2] Another result of crowding may be the spatial and temporal association of key enzymes involved in specific metabolic pathways, allowing for the coordinated passage of substrates and products within the colocalized enzyme system.

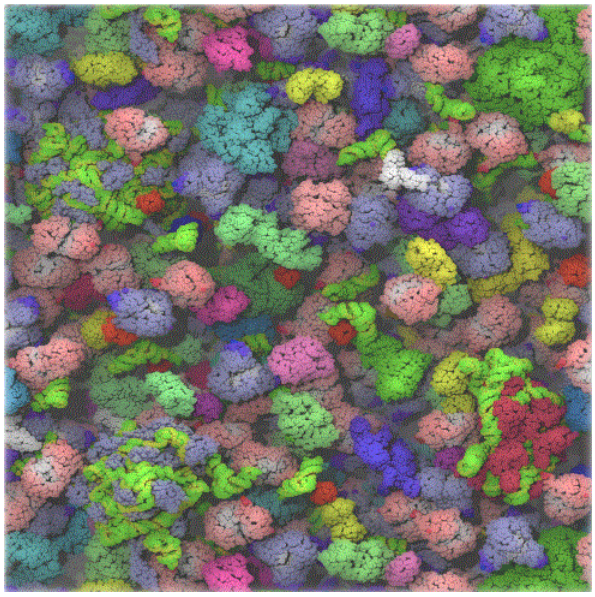


Figure 1.5: The Crowded Cytoplasm of E. Coli. The computer simulation used 50 different types of the most abundant macromolecules of the E. coli cytoplasm and 1008 individual molecules. Rendering of the cytoplasm model at the end of a dynamics simulation. RNA is shown as green and yellow. This figure was prepared with VMD. Figure adapted from: Ufrom McGuffee SR, Elcock AH (2010) PLoS Comput Biol 6(3): e1000694. doi:10.1371/journal.pcbi.1000694 (open source journal)

Cell components undergo phase transitions to form substructures within the cell.

A perplexing question is how substructures form within a cell. This includes the biogenesis of organelles like mitochondria and smaller particles such as polysaccharide granules, lipid droplets, protein/RNA particles (including the ribosome), and the nucleolus of the cell nucleus. It might be easiest to consider this problem using two examples from the lipid world: lipid droplets and membrane rafts. Phase transitions occur when a sparingly soluble nonpolar liquid is added to water. At a high enough concentration, the solubility of the nonpolar liquid is exceeded, and a phase transition occurs, as evidenced by the appearance of two separate liquid phases. The identical process occurs when triglycerides coalesce into lipid droplets with proteins associated on their outside. Another example occurs within a cell membrane when lipids with saturated alkyl chains self-associate with membrane cholesterol (which contains a rigid planar ring system) to form a lipid raft membrane microdomain***. Lipid rafts*** are characterized by greater packing efficiency, rigidity, and thickness than other membrane parts. These lipid rafts often recruit proteins involved in signaling processes within the cell membranes. This phase separation process is called ***liquid/liquid demixing,*** as two “liquid-like” substances separate.

Similarly, it appears that proteins that interact with RNA are composed of less diverse amino acid sequences and have more flexible (“more liquid-like) structures, allowing their preferential interaction with RNA to form large RNA-protein particles (like the ribosome and other RNA processing structures) in a fashion that mimics liquid/liquid demixing. All of these interactions are just manifestations of the various intermolecular forces that can exist between molecules. These include ionic interactions, ion-dipole interactions, dipole-dipole interactions, and London dispersion forces. A review of intermolecular forces can be found in a Kahn Academy video on YouTube.

Summary

Chapter Summary

This chapter offers an integrative overview of cellular biochemistry by framing the cell as a dynamic chemical factory. It emphasizes that, although cells have been traditionally studied from a biological perspective, a chemical viewpoint reveals the intricate processes that drive cellular life. Key points include:

- **Cellular Structure and Biomolecules:**
  The chapter begins by reviewing the fundamental organization of cells, distinguishing between prokaryotic and eukaryotic types. It highlights the common presence of major biomolecules—lipids, proteins, nucleic acids, and carbohydrates—and outlines their synthesis, distribution, and degradation within eukaryotic animal cells.

- **Metabolic Processes:**
  Metabolism is portrayed as the orchestrated series of chemical reactions that power cellular functions. This includes:
  - **Catabolic Reactions:** Breaking down complex molecules to release energy.
  - **Anabolic Reactions:** Building complex biomolecules from simpler units using energy.
  - The integration of these processes is crucial for energy balance, growth, and response to environmental changes.
- **Enzyme Catalysis and Regulation:**
  Enzymes are introduced as essential catalysts that accelerate metabolic reactions. Two primary models of enzyme-substrate interactions are discussed:
  - **Lock and Key Model:** Emphasizing a perfect fit between enzyme and substrate.
  - **Induced Fit Model:** Highlighting structural changes in the enzyme upon substrate binding. These models illustrate how enzyme structure is key to function and regulation, allowing cells to respond to internal and external signals.
- **Cellular Compartmentalization and Transport:**
  The chapter explains how the compartmentalization of metabolic processes into distinct organelles (such as the nucleus, mitochondria, endoplasmic reticulum, and lysosomes) enhances efficiency and regulation. It also covers various transport mechanisms—including passive diffusion, facilitated diffusion, and active transport—that allow selective import and export of molecules, thereby maintaining internal homeostasis.
- **Cellular Architecture and Crowding:**
  An analogy to a factory is used to describe the cytoskeleton—a network of microfilaments, intermediate filaments, and microtubules—that provides structural support and facilitates intracellular transport. The crowded cellular environment is noted for influencing biomolecular interactions, protein stability, and localized reaction dynamics.
- **Phase Transitions and Subcellular Organization:**
  Finally, the chapter discusses how phase separation phenomena, such as liquid/liquid demixing, lead to the formation of specialized cellular substructures. Examples include the formation of lipid droplets and membrane rafts, which are driven by intermolecular forces like ionic and dipole interactions.

Overall, the chapter sets the stage for deeper exploration into the structure, function, and regulation of specific biomolecules by connecting fundamental chemical principles with cellular physiology and highlighting their relevance to health and disease.

1.2: Chemical Foundations

Learning Goals (ChatGPT o3-mini)

1. **Fundamental Concepts in Organic Chemistry**
   - Define what constitutes an organic molecule and distinguish it from inorganic compounds.
   - Explain the significance of carbon's versatility in forming millions of unique compounds.
2. **Understanding Functional Groups**
   - Identify and describe common functional groups (e.g., hydroxyl, carbonyl, carboxyl, amine, phosphate, etc.) and their characteristic bonding patterns.
   - Predict the reactivity of a molecule based on the presence and arrangement of its functional groups.
3. **Classification of Hydrocarbons**
   - Differentiate between alkanes, alkenes, alkynes, and aromatics in terms of structure and reactivity.
   - Explain the concepts of saturation versus unsaturation and the implications for chemical behavior.
4. **Reactivity and Structural Diversity**
   - Illustrate how similar functional groups in different molecules lead to predictable reactivity patterns.
   - Analyze examples (such as capsaicin) to identify multiple functional groups within a single compound and discuss their collective impact on molecular behavior.
5. **Specialized Functional Group Classes**
   - Distinguish between closely related groups such as alcohols, phenols, and thiols, as well as ethers versus sulfides.
   - Understand the classification (primary, secondary, tertiary) of both alcohols and amines, noting how definitions differ between these groups.
6. **Organic Phosphates and Carbonyl Compounds**
   - Explain the structural and functional roles of organic phosphates in biomolecules.
   - Compare and contrast aldehydes, ketones, and carboxylic acids along with their derivatives, focusing on their reactivity in biological contexts.
7. **Linking Organic Chemistry to Biochemistry**
   - Demonstrate how functional groups participate in dehydration synthesis reactions that form major macromolecules (proteins, nucleic acids, carbohydrates, and lipids).
   - Recognize the importance of functional group activation in the synthesis of complex biomolecules.
8. **Primary Metabolites and Cellular Function**
   - Define primary metabolites and explain their role in fundamental cellular processes such as energy production, growth, and signaling.
   - Relate the chemical principles of organic reactions to the formation and function of cellular structures (e.g., membranes, cell walls, cytoskeleton).

By achieving these goals, students will deepen their understanding of the structure, reactivity, and biological significance of organic molecules and functional groups, laying a strong foundation for exploring more complex biochemical processes.

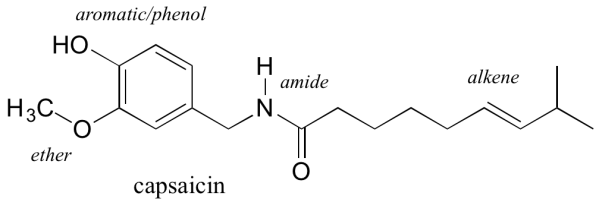
1.2.1: Organic Molecules

On Earth, all carbon-containing molecules have originated from biological, living organisms and are called **organic compounds**. The number of known organic compounds is quite large. There are many times more organic compounds known than all the other (inorganic) compounds discovered, about 7 million organic compounds. Fortunately, organic chemicals consist of relatively few similar parts combined differently. These structural similarities allow us to predict how a compound we have never seen before may react if we know how other molecules containing the same types of parts are known to react.

These parts of organic molecules are called **functional groups** with specific bonding patterns and atoms most commonly found in organic molecules (C, H, O, N, S, and P). Identifying functional groups and predicting reactivity based on functional group properties is one of the cornerstones of organic chemistry. Functional groups are specific atoms, ions, or groups of atoms having consistent properties. A functional group makes up part of a larger molecule. For example, -OH, the hydroxyl group that characterizes alcohols, contains oxygen with attached hydrogen. It could be found on any number of different molecules. Just as elements have distinctive properties, functional groups have characteristic chemistries. An -OH functional group on one molecule will tend to react similarly, although perhaps not identically, to an -OH on another molecule.

Organic reactions usually occur in the functional group, so learning about the reactivities of functional groups will prepare you to understand many other aspects of biochemistry.

Functional groups are structural units within organic compounds defined by specific bonding arrangements between specific atoms. The structure of capsaicin, the fiery compound found in hot peppers, has several functional groups, labeled in the figure below and explained throughout this section.

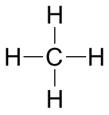


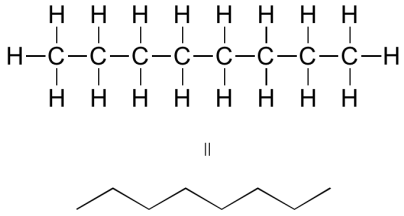
capsaicin

As we progress in the study of biochemistry, it will become extremely important to recognize the most common functional groups. These *are the key structural elements that define how organic molecules react*. Below is a brief introduction to the major organic functional groups.

1.2.1.1: Alkanes

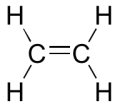
The 'default' in organic chemistry (the *lack* of functional groups) is given the term *alkane*, characterized by single bonds between carbon and carbon or carbon and hydrogen. Methane, CH_4, is the natural gas you may burn in your furnace. Octane, C_8H_{18}, is a component of gasoline.

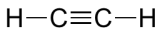

methane


octane

1.2.1.2: Alkenes and Alkynes

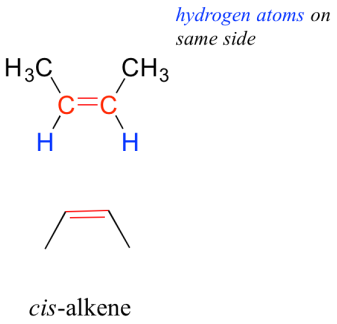
Alkenes (sometimes called olefins) have carbon-carbon double bonds, and *alkynes* have carbon-carbon triple bonds. Ethene, the simplest alkene example, is a gas that serves as a cellular signal in fruits to stimulate ripening. (If you want bananas to ripen quickly, put them in a paper bag along with an apple – the apple emits ethene gas (also called ethylene), setting off the bananas' ripening process)). Ethyne, commonly called acetylene, is used as a fuel in welding blow torches.


ethene
(an alkene)

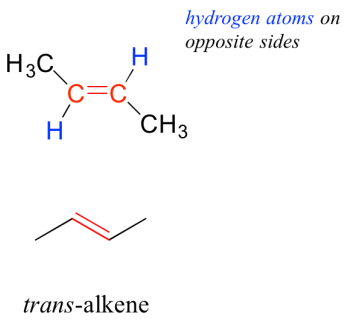

ethyne
(an alkyne)

Many alkenes can take two geometric forms: *cis* or *trans*. The *cis* and *trans* forms of a given alkene are different isomers with different physical properties because there is a very high energy barrier to rotation about a double bond. In the example below, the

difference between *cis* and *trans* alkenes is readily apparent. Biochemists don't usually use the E (entgegen) and Z (zusammen) labels for groups attached to double bonds (using IUPAC priority numbering).



cis-alkene

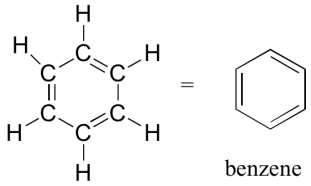


trans-alkene

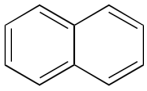
Alkanes, alkenes, and alkynes are all classified as *hydrocarbons* because they are composed solely of carbon and hydrogen atoms. Alkanes are said to be *saturated hydrocarbons* because the carbons are bonded to the maximum possible number of hydrogens – in other words, they are '*saturated*' with hydrogen atoms. The double and triple-bonded carbons in alkenes and alkynes have fewer hydrogen atoms bonded to them – they are thus referred to as *unsaturated hydrocarbons*.

1.2.1.3: Aromatics

The *aromatic group* is exemplified by benzene and naphthalene, a compound with a distinctive 'mothball' smell. Aromatic groups are planar (flat) ring structures with conjugated pi bonding with 4n+2 pi electrons. Given the stability of aromatic groups due to the delocalization of the pi electrons, these groups are widespread.



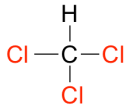
benzene



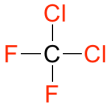
napthalene

1.2.1.4: Alkyl Halides

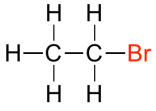
When the carbon of an alkane is bonded to one or more halogens, the group is referred to as an *alkyl halide or haloalkane*. Chloroform is a valuable solvent in the laboratory and was one of the earlier anesthetic drugs used in surgery. Chlorodifluoromethane was used as a refrigerant and in aerosol sprays until the late twentieth century. Still, its use was discontinued after it was found to have harmful effects on the ozone layer. Bromoethane is a simple alkyl halide often used in organic synthesis. Alkyl halide groups are quite rare in biomolecules.



trichloromethane (chloroform)



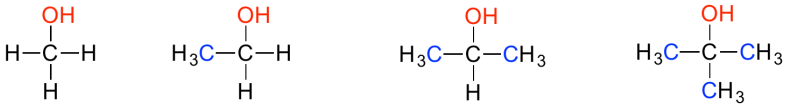
dichlorodifluoromethane (Freon-12)



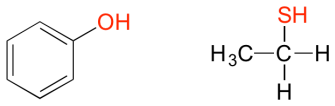
bromoethane

1.2.1.5: Alcohols, Phenols, and Thiols

In the ***alcohol*** functional group, a carbon is single-bonded to an OH group (the OH group, when it is part of a larger molecule, is referred to as a ***hydroxyl group***). All alcohols can be classified as primary, secondary, or tertiary. In primary alcohols, the carbon bonded to the OH group is also bonded to only one other carbon. The carbon is bonded to two or three other carbons in secondary and tertiary alcohols. When the hydroxyl group is *directly* attached to an aromatic ring, the resulting group is called a ***phenol.*** The sulfur analog of an alcohol is called a thiol (from the Greek *thio*, for sulfur).

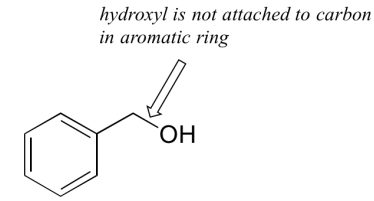


methanol | primary alcohol | secondary alcohol | tertiary alcohol



phenol | thiol

Note that the definition of a phenol states that the hydroxyl oxygen must be *directly* attached to one of the carbons of the aromatic ring. The compound below, therefore, is *not* a phenol – it is a primary alcohol.

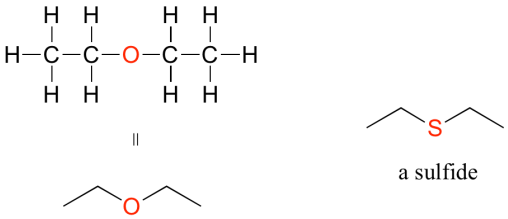


primary alcohol
(*not* a phenol)

The distinction is important because there is a significant difference in the reactivity of alcohols and phenols.

1.2.1.6: Ethers and Sulfides

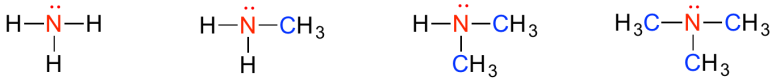
In an *ether* functional group, oxygen is bonded to two carbons. Below is the structure of diethyl ether, a common laboratory solvent and one of the first compounds to be used as an anesthetic during operations. The sulfur analog of ether is called a *thioether or sulfide*.

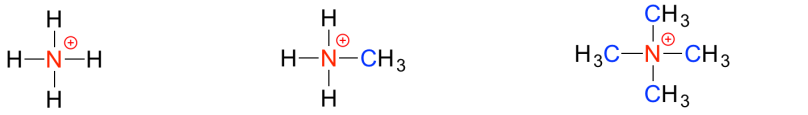


an ether | a sulfide

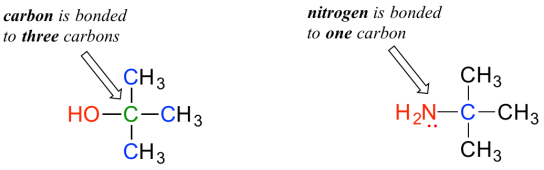
1.2.1.7: Amines

Amines contain nitrogen atoms with single bonds to hydrogen and carbon. Just as there are primary, secondary, and tertiary alcohols, there are primary, secondary, and tertiary amines. Ammonia is a special case with no carbon atoms. One of the most important properties of amines is that they are basic and are readily protonated to form ammonium cations. In the case where nitrogen has four bonds to carbon (which is somewhat unusual in biomolecules), it is called a quaternary ammonium ion.



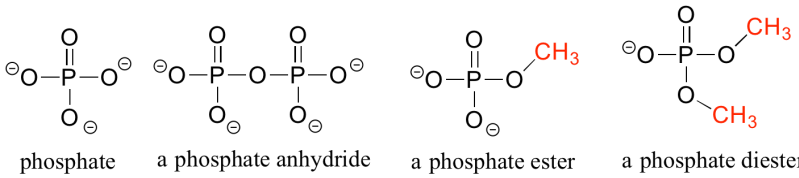


Note: Do not be confused by how the terms ‘primary’, ‘secondary’, and ‘tertiary’ are applied to alcohols and amines – the definitions are different. In alcohols, what matters is how many other carbons the alcohol *carbon* is bonded to, while in amines, what matters is how many carbons the *nitrogen* is bonded to.



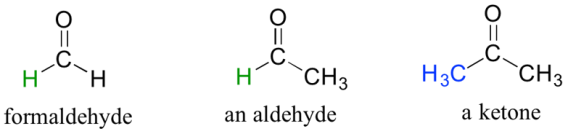
1.2.1.8: Organic Phosphates

Phosphate and its derivative functional groups are ubiquitous in biomolecules. Phosphate linked to a single organic group is called a *phosphate ester*; when it has two links to organic groups, it is called a phosphate diester. A linkage between two phosphates creates a phosphate anhydride.



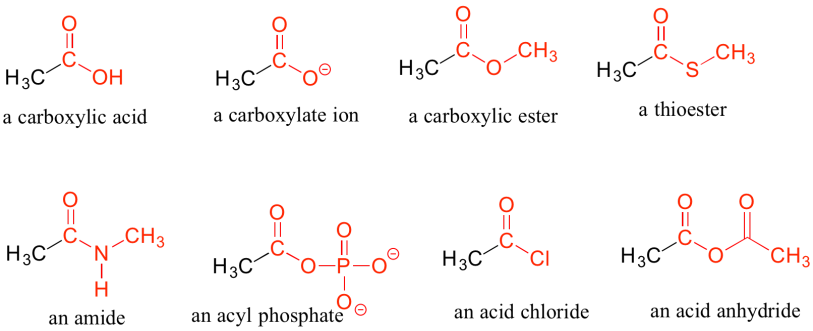
1.2.1.9: Aldehydes and Ketones

Many functional groups contain a carbon-oxygen double bond, which is commonly referred to as a *carbonyl*. Ketones and aldehydes are two closely related carbonyl-based functional groups that react similarly. In a *ketone*, the carbon atom of a carbonyl is bonded to two other carbons. In an *aldehyde*, the carbonyl carbon is bonded on one side to hydrogen and on the other side to carbon. The exception to this definition is formaldehyde, in which the carbonyl carbon has bonds to two hydrogens.



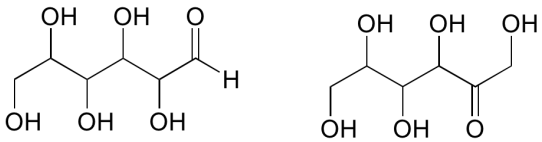
1.2.1.10: Carboxylic Acids and Their Derivatives

When a carbonyl carbon is bonded on one side to a carbon (or hydrogen) and on the other side to an oxygen, nitrogen, or sulfur, the functional group is considered to be one of the carboxylic acid derivatives. The main member of this family is the *carboxylic acid* functional group, in which the carbonyl is bonded to a hydroxyl group. The *carboxylate ion* form has donated the H^+ to the solution. Other derivatives are *carboxylic esters*(usually just called ‘esters’), *thioesters*, *amides*, *acyl phosphates*, *acid chlorides*, and *acid anhydrides*. Except for acid chlorides and acid anhydrides, carboxylic acid derivatives are very common in biological molecules and/or metabolic pathways and will be discussed in further detail in a later chapter.



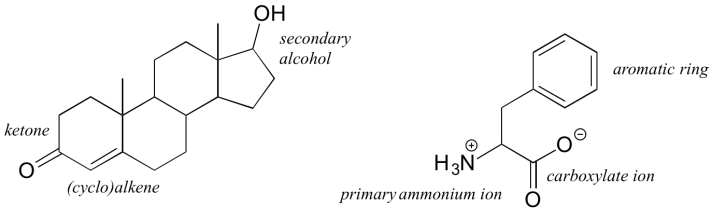
1.2.2: Practice Recognizing Functional Groups in Molecules

A single compound often contains several functional groups, particularly in biological organic chemistry. For example, the six-carbon sugar molecules glucose and fructose contain aldehyde and ketone groups, respectively, containing five alcohol groups. A compound with several alcohol groups is often referred to as a *'polyol'*.

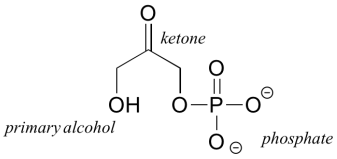


glucose fructose

The hormone testosterone, the amino acid phenylalanine, and the glycolysis metabolite dihydroxyacetone phosphate all contain multiple functional groups, as labeled below.



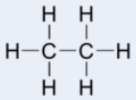
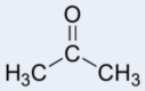
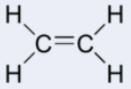
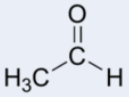
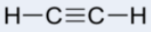
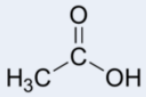
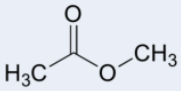
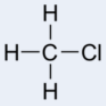
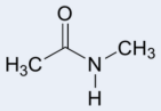
testosterone phenylalanine

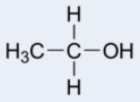
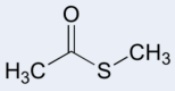
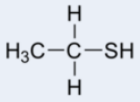
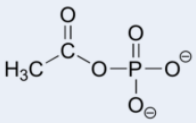
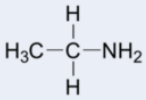
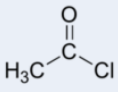
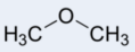
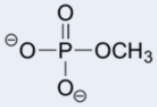
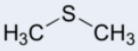
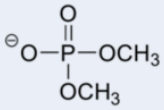
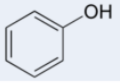
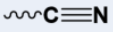


dihydroxyacetone phosphate
(DHAP)

While not a complete list, this section covers the most important functional groups we will encounter in biochemistry. Table 1.7 provides a summary of all the groups listed in this section.

Table 1.7 Common Organic Functional Groups

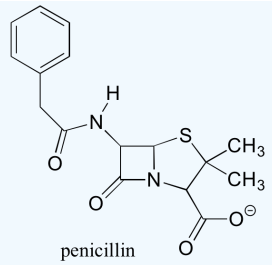
alkane		ketone	
alkene		aldehyde	
alkyne		carboxylic acid	
aromatic		ester	
alkyl halide		amide	

alcohol		thioester	
thiol		acyl phosphate	
amine		acid chloride	
ether		phosphate monoester	
thioether		phosphate diester	
phenol		nitrile	

Exercise 1.2.1

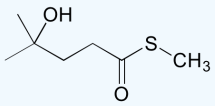
Identify the functional groups (other than alkanes) in the following organic compounds. State whether alcohols and amines are primary, secondary, or tertiary.

a)

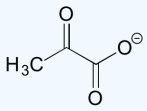


penicillin

b)

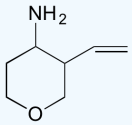


c)



pyruvate

d)



? Exercise 1.2.2

Draw one example of each compound fitting the descriptions below using line structures. Be sure to designate the location of all non-zero formal charges. All atoms should have complete octets (phosphorus may exceed the octet rule). There are many possible correct answers for these, so check your structures with your instructor or tutor.

a. a compound with molecular formula $C_6H_{11}NO$ that includes alkene, secondary amine, and primary alcohol functional groups.
b. an ion with molecular formula $C_3H_5O_6P^{2-}$ that includes aldehyde, secondary alcohol, and phosphate functional groups.
c. A compound with molecular formula C_6H_9NO that has an amide functional group and does *not* have an alkene group.

1.2.3: Primary metabolites

Primary metabolites are components of basic metabolic pathways required for life. They are associated with essential cellular functions such as nutrient assimilation, energy production, and growth/development. Primary metabolites include the building blocks required to make the four major macromolecules within the body: carbohydrates, lipids, proteins, and nucleic acids (DNA and RNA).

Large polymers are made from repeating smaller monomer units (Fig. 1.28). The nucleotides are the monomer units for building nucleic acids, DNA, and RNA. In contrast, the monomers for proteins are amino acids, carbohydrates are sugar residues, and lipids are fatty acids or acetyl groups.

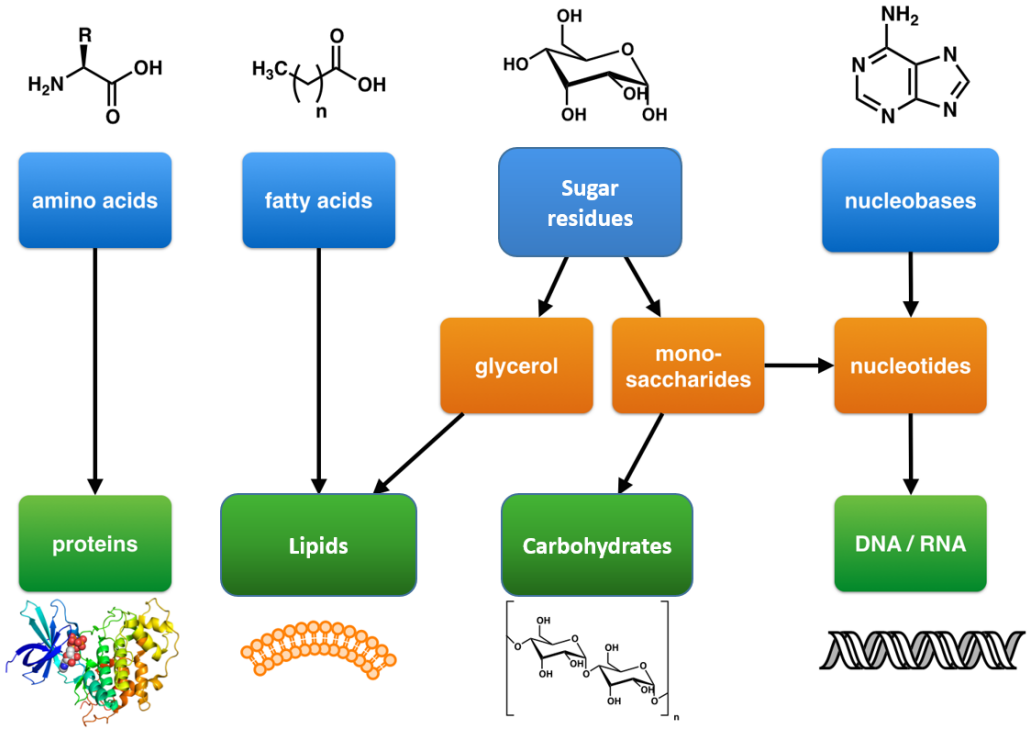


Figure 1.28 The Molecular building blocks of life are made from organic compounds. Modified from: Boghog

1.2.4: Reactions forming the Major Macromolecules

The major macromolecules are built by combining repeating monomer subunits through dehydration synthesis. Interestingly, the organic functional units used in the dehydration synthesis processes for each major macromolecule type have similarities. Thus, it is helpful to look at the reactions together (Figure 1.29)

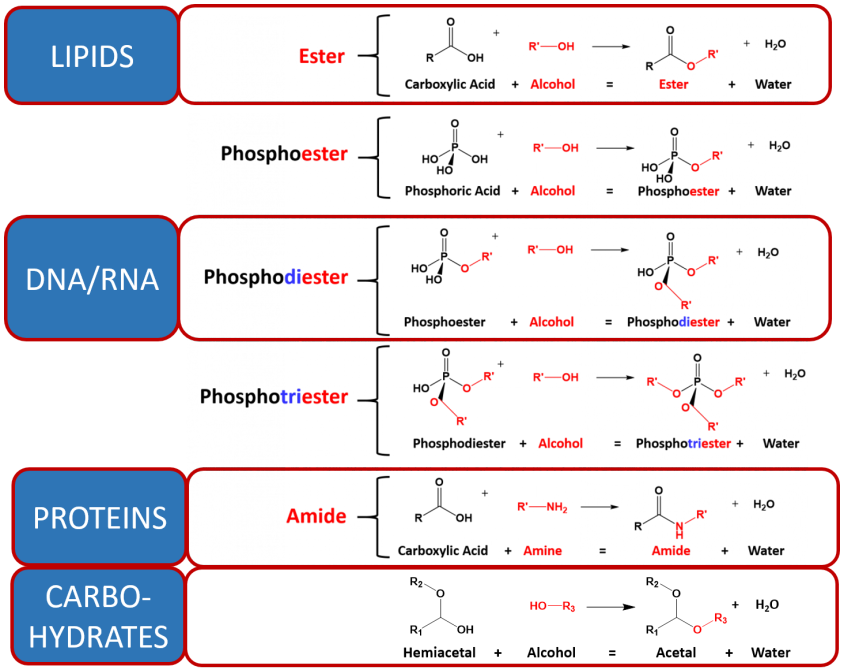


Figure 1.29 Dehydration Synthesis Reactions Involved in Macromolecule Formation. The major organic reactions required for the biosynthesis of lipids, nucleic acids (DNA/RNA), proteins, and carbohydrates are shown. Note that in all of these reactions, there is a functional group that contains two electron-withdrawing groups (the carboxylic acid, phosphoric acid, and the hemiacetal, each having two oxygen atoms attached to a central carbon or phosphorus atom). This forms a reactive, partially positive center atom (carbon in the case of the carboxylic acid and hemiacetal, or phosphorus in the case of the phosphoric acid) that the electronegative oxygen or nitrogen can attack from an alcohol or amine functional group. Within biological systems, many functional groups, such as carboxylic acids, require activation before they can be utilized in synthesis reactions, which will be detailed in later chapters.

Primary metabolites involved with energy production include numerous enzymes that break down food molecules, such as carbohydrates and lipids, and capture the energy released during the hydrolysis of adenosine triphosphate (ATP). *Enzymes* are biological catalysts that speed up the rate of chemical reactions. Typically, they are proteins, which are composed of amino acid building blocks. Cells are also composed of primary metabolites. These include cell membranes (e.g., phospholipids), cell walls (e.g., peptidoglycan, chitin), and cytoskeletons (proteins). DNA and RNA, which store and transmit genetic information, comprise nucleic acid primary metabolites. Primary metabolites also also involved in cellular signaling, communication, and transport molecules. The structure and function of primary metabolites are key components of this text. These reactions will be detailed in the following chapters.

1.2.5: Summary

This chapter introduces the essential concepts of organic chemistry as they apply to biochemistry, highlighting the central role that carbon-containing compounds play in all life on Earth. It emphasizes that, despite the enormous diversity of organic compounds—numbering in the millions—they are composed of a limited number of recurring structural units known as functional groups. Understanding these groups is fundamental because their predictable reactivity patterns allow us to infer the chemical behavior of unfamiliar compounds.

The chapter begins by discussing the origins of organic molecules and explains how the unique bonding capabilities of carbon allow for the formation of complex and diverse molecular architectures. It outlines how functional groups such as hydroxyl, carbonyl, carboxyl, amine, phosphate, and others serve as the "building blocks" that determine the reactivity and interactions of organic molecules. This foundational knowledge is critical for predicting reaction outcomes in both synthetic organic chemistry and biological systems.

A significant portion of the chapter is dedicated to classifying organic compounds based on their structural features. It covers simple hydrocarbons, distinguishing between alkanes (saturated hydrocarbons), alkenes and alkynes (unsaturated hydrocarbons with double and triple bonds, respectively), and aromatic compounds known for their stable, conjugated ring structures. In addition,

the chapter explores various derivatives formed when functional groups are introduced, such as alkyl halides, alcohols, phenols, thiols, ethers, and amines—each with its own characteristic reactivity and role in biological systems.

Further, the chapter connects these organic chemistry principles to biochemistry by explaining how dehydration synthesis reactions link monomers into macromolecules. It details the construction of proteins, nucleic acids, carbohydrates, and lipids from their respective monomer units and discusses the importance of functional group activation during these biosynthetic processes. The section on primary metabolites ties together the concept of organic reactivity with cellular function, underscoring the essential roles of these molecules in energy production, cell structure, and signaling.

Lastly, exercises included in the chapter challenge students to apply their understanding by identifying functional groups in complex molecules and designing compounds that meet specific structural criteria. This active learning component reinforces the practical importance of functional groups in predicting and controlling chemical reactivity in biological contexts.

Overall, the chapter equips students with a robust framework for understanding the structure and reactivity of organic molecules, laying the groundwork for more advanced studies in biochemistry and molecular biology.

1.3: Physical-Chemical Foundations

Learning Goals (ChatGPT o3-mini)

1. **Understand the Driving Forces of Reactions**
   - Explain how energy differences (ΔG) between reactants and products dictate the spontaneity and extent of chemical reactions.
   - Use the “ball on a hill” analogy to relate potential energy differences to reaction spontaneity.
2. **Distinguish Reversible and Irreversible Reactions**
   - Describe the characteristics of irreversible reactions versus reversible reactions and illustrate how reaction progress and equilibrium are reflected in concentration-time profiles.
   - Interpret different scenarios (favoring products, favoring reactants, or balanced) based on changes in reactant and product concentrations.
3. **Apply Equilibrium Concepts**
   - Define the equilibrium constant (K_{eq}) and explain how it quantitatively reflects the relative favorability of reactants versus products.
   - Relate the value of K_{eq} (greater than, less than, or equal to 1) to the extent of reaction and spontaneous direction.
4. **Analyze the Role of Concentration in Reaction Energetics**
   - Differentiate between the intrinsic stability of reactants/products (ΔG^o) and the influence of concentration on reaction spontaneity via the reaction quotient (Q_{rx}).
   - Explain how changing concentrations (as per Le Chatelier’s Principle) can shift equilibrium positions.
5. **Calculate and Interpret Gibbs Free Energy Changes**
   - Use the relationship $\Delta G = \Delta G^o + RT \ln Q$ to calculate the free energy change under non-standard conditions.
   - Discuss how the sign and magnitude of ΔG determine whether a reaction proceeds toward products, remains at equilibrium, or reverses.
6. **Connect Thermodynamic Parameters: Enthalpy and Entropy**
   - Define enthalpy (ΔH) and entropy (ΔS) and explain their roles in determining the spontaneity of both exothermic and endothermic reactions.
   - Compare and contrast exothermic ($\Delta H < 0$) and endothermic ($\Delta H > 0$) processes in the context of biological reactions.
7. **Integrate the Second Law of Thermodynamics in Biochemical Contexts**
   - Explain the significance of the second law of thermodynamics ($\Delta S\_total > 0$) and how it governs spontaneous processes.
   - Relate macroscopic thermodynamic changes (ΔG_sys) to microscopic rearrangements and energy dispersion (ΔS_{sys} and ΔS_{surr}) in biochemical systems.
8. **Apply Thermodynamic Principles to Real-World Biochemical Reactions**
   - Analyze case studies, such as the reactions of strong versus weak acids with water, to illustrate how intrinsic molecular stability and concentration effects combine to determine reaction direction and extent.
   - Evaluate the thermodynamic rationale behind phenomena like water evaporation and molecular binding, and discuss how these principles underpin cellular metabolism.

By achieving these learning goals, students will be equipped to analyze and predict the behavior of biochemical reactions, understand the interplay between energy, equilibrium, and entropy, and apply these concepts to complex biological systems.

The types and numbers of chemical reactions in biological cells are staggering. Compared to physical and chemical reactions in a controlled and closed environment, biological reactions occur in open systems with input and output of energy and chemical "feedstocks." Yet, they are governed by the same physical principles that control all reactions. We can gain insight into biological

reactions and how they are controlled by considering the principles you have used in many preceding classes: energy changes, equilibria, and thermodynamics. Let's review them!

1.3.1: Reactions and Energy Changes

Why do reactions vary in extent from completely irreversible in the forward reaction to reversible reactions favoring the reactants? It might help to understand a simple physical reaction before we try more complicated chemical reactions. Let's start with a simple ball on a hill. Does a ball at the top of a hill roll downhill spontaneously, or does the opposite happen? No one has ever seen a ball roll spontaneously uphill unless a lot of energy was added to the ball. This physical reaction appears irreversible and occurs because the ball has lower potential energy at the bottom of the hill than it does at the top. The gap in the potential energy is related to the "extent" and spontaneity of this reaction. As we have undoubtedly observed, natural processes tend to go to a lower energy state. By analogy, we will consider the driving force for a chemical reaction to be the free energy difference, ΔG, between reactants and products. ΔG determines the extent and spontaneity of the reaction.

1.3.1.1: Reversible/Irreversible Reactions, Extent of Reactions, Equilibria

Consider a hypothetical reversible reaction in which you start with some reactants, A and B, each at a 1 M concentration (1 mol of each/L solution). but no products, P and Q. For ease, assume that the total volume of solution is 1 L, so that we start with 1 mol each of A and B. At time $t = 0$, the concentration of products is 0. The reaction can be written as:

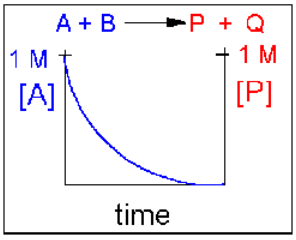
$$\mathrm{A} + \mathrm{B} \rightleftharpoons \mathrm{P} + \mathrm{Q}.$$

As time progresses, the amounts or concentrations of A and B decrease as the amounts or concentrations of products P and Q increase. At some time, no further changes occur in the amount or concentrations of remaining reactants or products. At this point, the reaction is in equilibrium, a term often used in our common vocabulary to denote a system undergoing no net change.

Most of the reactions we will study occur in solution, so we will deal with concentrations (mol/L or mmol/mL = M). Let's consider how the concentration of reactants and products changes as a function of time. Depending on the extent to which a reaction is reversible, four different scenarios can be imagined:

Scenario 1: Irreversible reaction in which the reverse reaction occurs to a negligible extent.

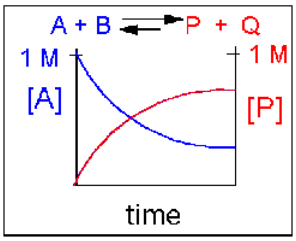
In this reaction, the reverse reaction occurs to such a small extent that we can neglect it. The only reaction that occurs is the conversion of reactants to products. Hence, all the reactants are converted to product. At equilibrium [A] = 0. Since 1 mol of A reacted, it must form 1 mol of P and 1 mol Q - i.e., the concentration of products at equilibrium is 1 M. At an earlier time of the reaction (let's pick a time when [A] = 0.8 M), only part of the reactants have reacted (in this case 0.2 M), producing an equal amount of products, P and Q. Graphs of [A] and [P] as a function of time are shown below. [A] decreases nonlinearly to 0 M while [P] increases reciprocally to 1 M concentration. This is illustrated in the graph below.



Examples of irreversible reactions are reactions of strong acids (nitric, sulfuric, hydrochloric) with bases (OH^- and water) or the much more complicated combustion reactions, such as the burning of sugars (like trees) and hydrocarbons (like octane) to form CO_2 and H_2O.

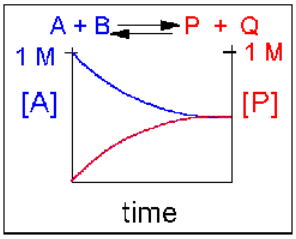
Scenario 2: Reversible reaction in which the forward reaction is favored.

Again, [A] decreases and [P] increases, but in this case, some A remains since the reaction is reversible. As [A]and [B] decrease, [P] and [Q] increase, which increases the chance that they will collide and form the product. Since P and Q can react to form reactants, the [A] at equilibrium is not zero, as is shown below.



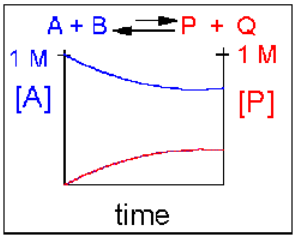
Scenario 3: Reversible Reaction in which forward and reverse reactions are equally favored.

Again, [A] decreases and [P] increases, but in this case, some A remains since the reaction is reversible. As [A] and [B] decrease, [P] and [Q] increase, which increases the chance that they will collide and form the product. Since P and Q can react to form reactants, the [A] at equilibrium is not zero, as is shown below. Because the reactants and products are equally favored, their concentrations will be equal at equilibrium.



Scenario 4: Reversible Reaction in which the reverse reaction is favored.

Again, [A] decreases, and [P] increases, but in this case, some A remains since the reaction is reversible. As [A] and [B] decrease, [P] and [Q] increase, which increases the chance that they will collide and form the product. Since P and Q can react to form reactants, the [A] at equilibrium is not zero, as shown below. Because the reaction favors reactants, their concentration will be higher at equilibrium than the products.



An example of this kind of reaction, one that favors reactants, is the reaction of acetic acid (a weak acid) with water.

$$\mathrm{CH_3CO_2H(aq) + H_2O(l) \rightleftharpoons H_3O^+(aq) + CH_3CO_2^-(aq)}$$

1.3.2: Equilibrium Constants

Without much experience in chemistry, it is difficult to just look at the reactants and products and determine whether the reaction is irreversible or reversible, favoring either reactants or products (except obvious irreversible reactions described above). However, this data can be found in tables of equilibrium constants. The equilibrium constant, as its name implies, is constant, independent of the concentration of the reactants and products. A $K_{eq} > 1$ implies that the products are favored. A $K_{eq} < 1$ implies that reactants are favored. Reactants and products are equally favored when $K_{eq} = 1$. For the more general reaction,

$$\mathrm{aA + bB \rightleftharpoons pP + qQ}$$

where a, b, p, and q are the stoichiometric coefficients,

$$\mathrm{K_{eq} = \frac{[P]_{eq}^{p}[Q]_{eq}^{q}}{[A]_{eq}^{a}[B]_{eq}^{b}}} \tag{1.3.1}$$

where all the concentrations are at their equilibrium values. For a simple reaction where a, b, p, and q are all 1, then $K_{eq} = ([P][Q])/([A][B])$.

(Note: Equilibrium constants are truly constant only at a given temperature, pressure, and solvent condition. Likewise, they depend on concentration to the extent that their activities change with concentration.)

For an irreversible reaction, such as the reaction of a 0.1 M HCl (aq) in water, $[HCl]_{eq}$ = 0, you cannot easily measure a K_{eq}. However, if we assume the reaction goes in reverse to an almost imperceptible degree, $[HCl]_{eq}$ might equal 10-10 M. Hence K_{eq} >> 1.

In summary, the extent of reactions can vary from completely irreversible (favoring only the products) to reactions that favor the reactants.

Our next goal is to understand what controls the extent of a reaction. That is, of course, the change in the Gibbs free energy. Two different pairs of factors influence the ΔG. One pair is the reactants' concentration and inherent reactivity compared to products (as reflected in the Keq). The other pair is enthalpy/entropy changes. We will now consider the first pair.

Contributions of Molecule Stability (K_{eq}) and Concentration to ΔG

Consider the reactions of hydrochloric acid and acetic acid with water.

$$\mathrm{HCl(aq) + H_2O(l) \longrightarrow H_3O^+(aq) + Cl^-(aq)}$$

$$\mathrm{CH_3CO_2H(aq) + H_2O(l) \longrightarrow H_3O^+(aq) + CH_3CO_2^-(aq)}$$

Assume that at t = 0, each acid is placed into water at a concentration of 0.1 M. When equilibrium is reached, essentially no HCl remains in the solution. In contrast, 99% of the acetic acid remains. Why are they so different? We rationalized that HCl (aq) is a much stronger acid than H_3O^+(aq) which itself is a much stronger acid than CH_3CO_2H (aq). Why? Something about the structure of these acids (and the bases) makes HCl much more intrinsically unstable, much higher in energy, and hence much more reactive than the acid it forms, H_3O^+(aq). Likewise, H_3O^+(aq) is much more intrinsically unstable, much higher in energy, and hence more reactive than CH_3CO_2H (aq). This has nothing to do with concentration since the initial concentration of both HCl (aq) and CH_3CO_2H (aq) were identical. This observation is reflected in the K_{eq} for these acids (>>1 for HCl and <<1 for acetic acid). This difference in intrinsic stability of reactants compared to products (which is independent of concentration) is one factor that contributes to ΔG.

The other factor is concentration. A 0.25 M (0.25 mol/L or 0.25 mmol/ml) solution of acetic acid does not conduct electricity, implying that very few ions of H_3O^+(aq) + $CH_3CO_2^-$ (aq) exist in the solution. However, a dim light becomes evident if more concentrated acetic acid is added. Adding more reactants seemed to drive the reaction to form more products, even though the reverse reaction is favored if one considers only the intrinsic stability of reactants and products. Before the concentrated acid was added, the system was at equilibrium. Adding concentrated acid perturbed the equilibrium, which drove the reaction to form additional products. This is an example of Le Chatelier's Principle, which states that if a reaction at equilibrium is perturbed, the reaction will be driven in the direction that will relieve the perturbation. Hence:

- if more reactant is added, the reaction shifts to form more products
- if more product is added, the reaction shifts to form more reactants
- if products are selectively removed (by distillation, crystallization, or further reaction to produce another species), the reaction shifts to form more product.
- if reactants are removed (as above), the reaction shifts to form more reactants.
- if heat is added to an exothermic reaction, the reaction shifts to remove the excess heat by shifting to form more reactants. (opposite for an endothermic reaction).

1.3.3: Change in Free Energy G

Without doing a complicated derivation, these simple examples suggest that the total ΔG can be expressed as the sum of the two contributions showing the effects of the intrinsic stability (K_{eq}) and concentration:

$$\Delta G_{\text{total}} = \Delta G_{\text{stability}} + \Delta G_{\text{concentration}} \tag{1.3.2}$$

which becomes for the simple reaction $\mathrm{A + B \rightleftharpoons P + Q}$ (after a rigorous derivation):

$$\begin{aligned} \Delta G &= \Delta G^0 + RT \ln \frac{[\mathrm{P}][\mathrm{Q}]}{[\mathrm{A}][\mathrm{B}]} \\ &= \Delta G^0 + RT \ln \mathrm{Q_{rx}} \end{aligned} \tag{1.3.3}$$

where ΔG^o reflects the contribution from the relative intrinsic stability of reactants and products, and the second term reflects the contribution from the relative concentrations of reactants and products (which has nothing to do with stability). Q_{rx} is the **reaction quotient**, which for the reaction A + B ↔ P + Q is given by:

$$Q_{rx} = ([P][Q])/([A][B]) \tag{1.3.4}$$

at any point in the reaction.

Meaning of ΔG

Remember that ΔG is the "driving" force for a reaction, analogous to the difference in potential energy for a ball on a hill. Go back to that analogy. If the ball starts at the top of the hill, does it roll downhill? Of course. It goes from high potential energy to low potential energy. The reaction can be written as:

$$\text{Ball}_{\text{top}} \rightarrow \text{Ball}_{\text{bottom}}$$

for which the change in potential energy, $\Delta PE = PE_{bottom} - PE_{top} < 0$. If the ball starts at the bottom, will it go to the top? Obviously not. For that reaction,

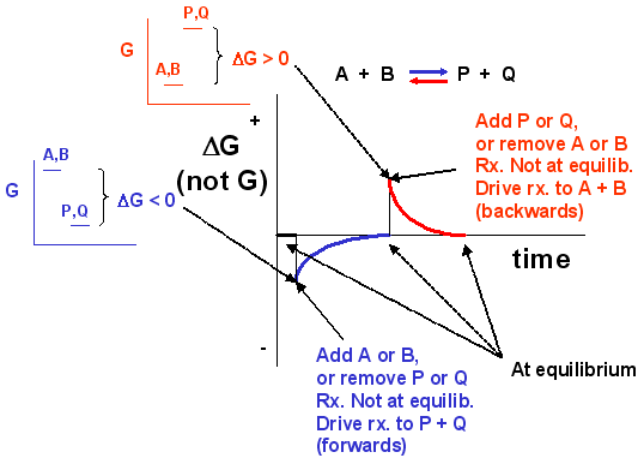
$$\text{Ball}_{\text{bottom}} \rightarrow \text{Ball}_{\text{top}}, \Delta PE > 0.$$

If the top of the hill were at the same height as the bottom of the hill (obviously an absurd situation), the ball would not move. It would effectively be at equilibrium, a state of no change. For this reaction, Ball_{top} --> $\text{Ball}_{\text{bottom}}$, the ΔPE = 0. As the ball starts rolling down the hill, its potential energy gets closer to the potential it would have at the bottom. Hence, the ΔPE changes from negative to more and more positive until it gets to the bottom, at which case the ΔPE = 0, and movement ceases. If the ΔPE is not 0, the ball will move until the ΔPE = 0.

Likewise, for a chemical reaction that favors products, ΔG < 0. The system is not at equilibrium, and the reaction will go toward products. As the reaction proceeds, products build up, and there is less of a driving force for reactants to go to products (Le Chatelier'sPrinciple), so the ΔG becomes more positive until the ΔG = 0 and the reaction is at equilibrium. A reaction with a ΔG > 0 is likewise not at equilibrium, so it will go in the appropriate direction until equilibrium is reached. Hence, for the reaction A + B <==> P + Q,

- if ΔG < 0, the reaction goes toward products P and Q
- if ΔG = 0, the reaction is at equilibrium, and no further change occurs in the concentration of reactants and products.
- if ΔG > 0, the reaction goes toward reactants A and B.

We can not measure easily the actual free energy G of reactants or products, but we can measure ΔG readily. These points are illustrated in the graph below of ΔG vs time for the hypothetical reaction A + B ↔ P + Q. (Also notice the two insert graphs - in blue and red - which show, in analogy to the ball on the hill graphs, the values of ΔG at the two points where the perturbation to the equilibrium was made.)



Notice the ΔG is constantly changing until the system reaches equilibrium. Initially, the equilibrium is perturbed so that the system is not in equilibrium (shown in blue). The perturbation was such that the products were favored. After reaching equilibrium, the

system was perturbed again to favor the reverse reaction. Notice, in this case, the ΔG for the reaction as written: A + B ↔ P + Q is positive - i.e., it is not in equilibrium. Therefore, the reaction (as written) goes backward to reactants. It is important to realize that the reported ΔG is for the reaction as written.

Now let's apply $\Delta G = \Delta G^o + RT\ln Q = \Delta G^o + RT\ln([P][Q])/([A][B])$ to two reactions we discussed above:

- $HCl(aq) + H_2O(l) \leftrightarrow H_3O^+(aq) + Cl^-(aq)$
- $CH_3CO_2H(aq) + H_2O(l) \leftrightarrow H_3O^+(aq) + CH_3CO_2^-(aq)$

At time t=0, 0.1 mole of HCl and CH_3CO_2H were added to two different beakers. The forward reaction is favored at this point, but obviously to different extents. The RTln Q would be identical for both acids since each reactant is at 0.1 M, but no products exist. However, the ΔG^o is negative for HCl and positive for acetic acid since HCl is a strong acid. Hence at t=0, **ΔG** for the HCl reaction is much more negative than for acetic acid. This is summarized in the table below. The direction of the arrow shows if products (-->) or reactants (<---) are favored. The size of the arrow shows very approximately to what extent the ΔG term is favored

Reaction at t=0	ΔG^o	RTln Q	ΔG
$HCl(aq) + H_2O(l)$	---------->	---------->	---------->
$CH_3CO_2H(aq) + H_2O(l)$	<----------	---------->	->

Now, when equilibrium is reached, no net change occurs in the concentration of reactants and products, and ΔG = 0. In the case of HCl, there is just an infinitesimal amount of HCl left and 0.1 M of each product, so concentration favors HCl formation. However, the intrinsic relative stability of reactants and products still favors products. In the case of acetic acid, most acetic acid remains (0.099 M) with little product (0.001 M), so concentration favors products. However, the intrinsic relative stability of reactants and products still favors reactants. This is summarized in the table below.

Reaction at equlib.	ΔG^ostab	RTln Q	ΔG
$HCl(aq) + H_2O(l)$	---------->	<----------	favors neither, = 0
$CH_3CO_2H(aq) + H_2O(l)$	<----------	---------->	favors neither, = 0

Compare the two tables above (one at time t= 0 and the other at equilibrium). Notice:

- ΔG^o does not change in a given set of conditions since it has nothing to do with concentration.
- Only RTln Q changes during a reaction until equilibrium is achieved.

Meaning of ΔG^o

To get a better meaning of the significance of ΔG^o, let's consider the following equations under two different conditions:

$$\Delta G = \Delta G^0 + RT\ln\frac{[\mathrm{P}][\mathrm{Q}]}{[\mathrm{A}][\mathrm{B}]} = \Delta G^0 + RT\ln \mathrm{Q}_{\mathrm{rx}} \tag{1.3.5}$$

Condition I: Reaction at equilibrium, ΔG = 0

The equation reduces to:

$$\Delta G^0 = -RT\ln\frac{[\mathrm{P}]_{\mathrm{eq}}[\mathrm{Q}]_{\mathrm{eq}}}{[\mathrm{A}]_{\mathrm{eq}}[\mathrm{B}]_{\mathrm{eq}}} = -2.303\mathrm{R}T\log \mathrm{K}_{\mathrm{eq}} \tag{1.3.6}$$

This supports our idea that ΔG^o is independent of concentration since K_{eq} should also be independent of concentration.

Condition II: The concentration of all reactants and products is 1 M (standard state, assuming solution reaction)

The equation reduces to:

$$\Delta G = \Delta G^0 + RT \ln \frac{[1][1]}{[1][1]} = \Delta G^0 + 2.303 RT \log 1 = \Delta G^0$$

$$\begin{aligned} \Delta G &= \Delta G^o + RT \ln\left(\frac{[1][1]}{[1][1]}\right) \\ &= \Delta G^o + 2.303 RT \log 1 \\ &= \Delta G^o \end{aligned} \tag{1.3.7}$$

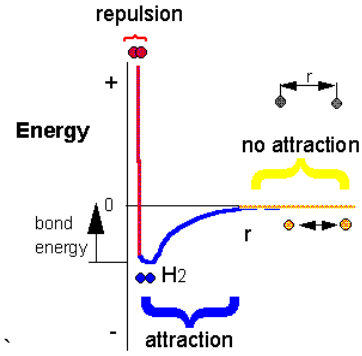
This implies that when all reactants are at this concentration, defined as the standard state (1 M for solutes), the ΔG at that particular moment is the ΔG^o for the reaction. If one of the reactants or products is H_3O^+, it would make little biological sense to calculate ΔG^o for the reaction using the standard state of $[H_3O^+]$ = 1 M or a pH of zero. Instead, it is assumed that the pH = 7, $[H_3O^+] = 10^{-7}$ M. A new symbol is used for ΔG^o under these conditions, $\Delta G^{o'}$.

1.3.4: Heat, Enthalpy and Entropy

Consider the association reaction of hydrogen atoms into molecular hydrogen

$$\mathrm{H + H \longrightarrow H_2}.$$

Does this reaction occur spontaneously? It does. You should remember that individual H atoms are unstable since they don't have a completed outer shell of electrons - in this case, a duet. As they approach, they can interact to form a covalent bond and, in the process, release energy. The bonded state is a lower energy state than two separated H atoms. This should be clear since energy has to be added to a molecule of H_2 to break the bond. We call this the **bond dissociation energy**.



Now consider a more complicated reaction, the burning of octane.

$$2\,\mathrm{C_8H_{18}(l)} + 25\,\mathrm{O_2(g)} \rightarrow 16\,\mathrm{CO_2(g)} + 18\,\mathrm{H_2O(g)}$$

To carry out this reaction, every C-C, C-H, and O-O bond in the reactants must be broken (which requires an input of energy), but lots of energy is released when the products' C-O and H-O covalent bonds are formed. Is more energy needed to break the bonds in the reactants, or is more energy released when forming bonds in the product? The answer should be clear. The products must be at a lower energy than the reactants since large amounts of heat and light energy are released from combustion of gasoline and other hydrocarbons.

These reactions suggest that energy must be released for a reaction to proceed to any extent in a given direction.

Now consider, however, the following reaction:

$$\mathrm{Ba(OH)_2 \cdot 8\,H_2O(s) + 2\,NH_4SCN(s) \longrightarrow 10\,H_2O(l) + 2\,NH_3(g) + Ba(SCN)_2(aq)}$$

When these two solids are added to a beaker and stirred, a reaction occurs, as evidenced by the formation of a liquid (water) and the smell of ammonia. Surprisingly, heat is not released into the surroundings in this reaction. Instead, heat was absorbed from the surroundings, turning the beaker so cold that it froze to a piece of wood (with a layer of water added to the wood) on which it was placed. This reaction seems to violate our idea that a reaction proceeds in a direction in which heat is liberated. Reactions that liberate heat and raise the temperature of the surroundings are called **exothermic** reactions. Reactions that absorb heat from the surroundings and, hence, lower the temperature of the surroundings are **endothermic** reactions. To answer the question, we need to consider entropy.

1.3.5: A review of thermodynamics

You may remember from General Chemistry that the change in the internal energy of a system, ΔE, is given by:

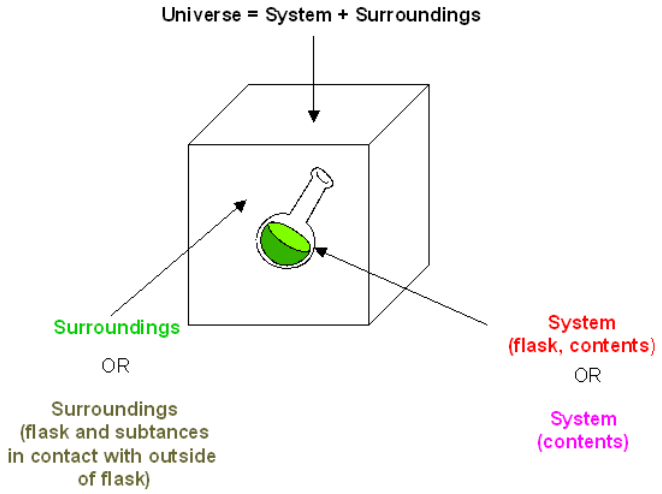
$$\begin{aligned} \Delta E_{sys} &= q + w \\ &= q - P_{ext}\,\Delta V \end{aligned} \tag{1.3.8}$$

where q is the heat (thermal energy) transferred to (+) or from the system (-), w is the work done on (+) or by (-) the system. This is one expression for the 1st Law of Thermodynamics

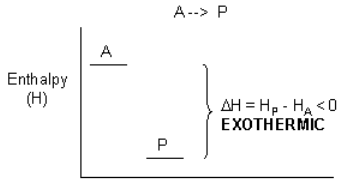
If only pressure/volume (PV) work is done (and not electrical work, for example), $w = -P_{ext}\,\Delta V$, where P_{ext} is the external pressure resisting a volume change in the system, ΔV. Under these conditions, the heat transfer at constant P, q_P is given by:

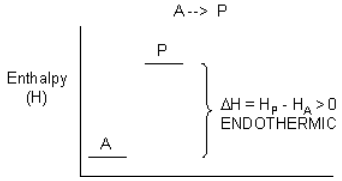
$$\begin{aligned} \Delta \mathrm{E_{sys}} - \mathrm{w} &= \Delta \mathrm{E_{sys}} + \mathrm{P_{ext}}\,\Delta \mathrm{V} \\ &= \mathrm{q_P} \\ &= \Delta \mathrm{H_{sys}} \end{aligned} \tag{1.3.9}$$

q_p, which can easily be measured in a coffee cup calorimeter, is equal to the **change in enthalpy**, ΔH, of the system.



For exothermic reactions, the reactants have more thermal energy than the products, and the heat energy (measured in kilocalories or kilojoules) released is the difference between the energy of the products and reactants. When heat energy is used to measure the difference in energy, we call the energy enthalpy (H) and the heat released as the change in enthalpy (ΔH), as illustrated below.





For exothermic reactions, $\Delta H < 0$. For endothermic reactions, $\Delta H > 0$.

The equation $\Delta \mathrm{E_{sys}} = \mathrm{q} + \mathrm{w} = \mathrm{q} - \mathrm{P_{ext}}\,\Delta \mathrm{V}$ is one expression of the First Law of Thermodynamics. Another statement of energy conservation is:

$$\Delta E_{tot} = \Delta E_{universe} = \Delta E_{sys} + \Delta E_{surrounding} = 0 \tag{1.3.10}$$

Something other than heat being released from the system decides whether a reaction proceeds to a significant extent. That is, the spontaneity of a reaction must depend on more than just ΔH_{sys}. Another example of a spontaneous natural reaction is the evaporation of water (a physical, not chemical, process).

$$H_2O(l) \rightarrow H_2O(g)$$

Heat is absorbed from the surroundings to break the intermolecular forces (H bonds) among the water molecules (the system), turning the liquid into a gas. If the surroundings are the skin, evaporating water in the form of sweat cools the body. Why are these reactions spontaneous and essentially irreversible even though they are endothermic? Notice that in both of these endothermic reactions (the reactions of $Ba(OH)_2.8H_2O(s)$ and $2NH_4SCN(s)$ and the evaporation of water), the products are more disorganized (more disordered) than the products. A solid is more ordered than a liquid or gas, and a liquid is more ordered than a gas. In nature, ordered things become more disordered with time. Entropy (S), the other factor (in addition to enthalpy changes), is often considered to measure a system's disorder. The greater the entropy, the greater the disorder. For reactions that go from order (low S) to disorder (high S), the change in S, $\Delta S > 0$. For the reaction that proceeds from low order to high order, $\Delta S < 0$.

Caution

However, this common description of entropy is quite misleading. Macroscopic examples describing order/disordered states (such as the cleanliness of your room or the shuffling of a deck of cards) are inappropriate since entropy deals with microscopic states.

The driving force for spontaneous reactions is the dispersion of energy and matter. Increases in entropy for reactions that involve matter occur when gases or solutes in solution are dispersed, leading to increases in positional entropy. For reactions involving energy changes, entropy increases when energy is dispersed as random, undirected thermal motion, leading to increases in thermal entropy. In this sense, entropy, S (a measure of ("spreadedness") is a measure of the number of different ways (microstates) that particles or energy can be arranged (W), not a measure of disorder! W is an abbreviation for the German word, ***Wahrscheinlichkeit***, which means probability. It can be shown that for a solute dissolving in a solvent, $W_{sys} = W_{solute} \times W_{solvent}$. Note that this is a multiplicative function. Entropy is a logarithmic function of W, which allows additivity of solute and solvent W values, a feature found in other thermodynamic state functions like ΔE, ΔH, and ΔS. Hence

$$\ln W \text{ sys} = \ln W_{solute} + \ln W_{solvent} \tag{1.3.11}$$

Boltzmann showed that for molecules,

$$S = k \ln W \tag{1.3.12}$$

where k is the Boltzmann constant (1.68×10^{-23} J/K), S units: J/K

or

$$S = kN_A \ln W = R \ln W \tag{1.3.13}$$

Boltzmann realized the connection between the macroscopic entropy of a system and the microscopic order/disorder of a system through the equation $S = k \ln W$. Increasing S (macroscopic property) occurs with increasing numbers of possible microscopic states for the atoms and molecules of a system.

The dissolution of a solute in water and the expansion of a gas into a vacuum, which both proceed spontaneously toward an increase in matter dispersal, are examples of familiar processes characterized by a $\Delta S_{sys} > 0$. We will see in future chapters that entropy changes in the solvent, solutes, and protein are critical determinants of protein folding.

The spontaneity of exothermic and endothermic processes will depend on the

$$\Delta S_{tot} = \Delta S_{surr} + \Delta S_{sys} \tag{1.3.14}$$

ΔS_{sys} often depends on matter dispersal (positional entropy). ΔS_{surr} depends on energy changes in the surroundings, $\Delta H_{surr} = -\Delta H_{sys}$ (thermal entropy).

It is more convenient to express thermodynamic properties based on the system that is being studied, not on the surroundings. This can be readily done for the $\Delta S_{surr,}$ which depends on ΔH_{sys} and the temperature. First, consider the dependency on ΔH_{sys}. Thermal

energy flows into or out of the system, and since $\Delta H_{sys} = - \Delta H_{surr}$,

ΔS_{surr} is proportional to $-\Delta H_{sys}$

- For an exothermic reaction, $\Delta S_{surr} > 0$ (since $\Delta H_{sys} < 0$) and the reaction is favored;
- For an endothermic reaction, $\Delta S_{surr} < 0$, (since $\Delta H_{sys} > 0$), and the reaction is disfavored;

ΔS_{surr} also depends on the temperature T of the surroundings:

ΔS_{surr} is proportional to 1/T

If the T_{surr} is high, a given heat transfer to or from the surroundings will have a smaller effect on the ΔS_{surr}. Conversely, if the T_{surr} is low, the effect on ΔS_{surr} will be greater. (Atkins uses the analogy of the effect of a sneeze in a library compared to a crowded street; An American Chemistry General Chemistry text uses the analogy of giving $5 to a friend with $1000 compared to one with just $10.) Hence,

$$\Delta S_{\text{surr}} = \frac{-\Delta \text{H}_{\text{sys}}}{\text{T}} \tag{1.3.15}$$

(Note: from a more rigorous thermodynamic approach, entropy can be determined from

$$\text{dS} = \frac{\text{dq}_{\text{rev}}}{\text{T}} \tag{1.3.16}$$

Once again,

$$\Delta S_{\text{tot}} = \Delta S_{\text{surr}} + \Delta S_{\text{sys}} \tag{1.3.17}$$

ΔS_{tot} depends on both enthalpy changes in the system and entropy changes in the surroundings. Hence,

$$\Delta S_{\text{tot}} = \frac{-\Delta \text{H}_{\text{sys}}}{\text{T}} + \Delta \text{S}_{\text{sys}} \tag{1.3.18}$$

Multiplying both sides by $-T$ gives

$$-\text{T}\Delta \text{S}_{\text{tot}} = \Delta \text{H}_{\text{sys}} + \text{T}\Delta \text{S}_{\text{sys}} \tag{1.3.19}$$

The thermodynamic function Gibb's Free Energy, G, can be defined as:

$$G = H - TS \tag{1.3.20}$$

At constant T and P,

$$\Delta G = \Delta H - T\Delta S \tag{1.3.21}$$

Hence

$$\Delta G_{sys} = \Delta H_{sys} - T\Delta S_{sys} = -T\Delta S_{tot} \tag{1.3.22}$$

Spontaneity is determined by ΔS_{tot} OR ΔG_{sys} since $\Delta S_{tot} = -\Delta G_{sys}/T$. ΔG_{sys} is widely used in discussing spontaneity since it is a state function, depends only on the enthalpy and entropy changes in the system, and is negative (as is the potential energy change for a falling object) for all spontaneous processes.

The second law of thermodynamics can be succinctly stated: For any spontaneous process, the $\Delta S_{tot} > 0$. Unlike energy (from the First Law), entropy is not conserved.

1.3.6: Summary

Chapter Summary: Reaction Energetics and Thermodynamics in Biological Systems

This chapter delves into the fundamental principles governing the vast array of chemical reactions that occur within biological cells. It begins by framing these reactions in the context of open systems, where both energy and chemical feedstocks flow in and out, yet the underlying behavior is dictated by the same thermodynamic laws that control all chemical processes.

Key Concepts and Analogies

- The chapter introduces the concept of free energy (ΔG) as the driving force of reactions, using the simple "ball on a hill" analogy to illustrate how reactions move from higher to lower energy states.
- It explains how the extent and spontaneity of reactions are determined by the free energy difference between reactants and products, much like a ball rolling downhill due to a drop in potential energy.

Reversible and Irreversible Reactions

- The discussion differentiates between irreversible reactions (where the reverse process is negligible) and reversible reactions, which can reach equilibrium.
- Four scenarios are described, ranging from reactions that go to completion (irreversible) to those where the equilibrium favors either the products, the reactants, or is balanced equally.

Equilibrium and the Reaction Quotient

- The equilibrium constant (K_eq) is introduced as a measure of the relative favorability of products versus reactants, with K_eq values greater than, less than, or equal to 1 indicating different reaction extents.
- The reaction quotient (Q_rx) is presented as a snapshot of the reaction state at any point in time, with its relationship to ΔG given by the equation:
  $\Delta G = \Delta G^0 + RT\ln Q_{rx}$

Intrinsic Stability Versus Concentration Effects

- The chapter distinguishes between ΔG^o, which reflects the intrinsic energy differences (or stability) of reactants and products, and the contribution of reactant/product concentrations.
- Through examples such as the reactions of HCl and acetic acid with water, it is shown that even when starting with equal concentrations, the inherent stability differences drive the reaction to different extents.

Thermodynamics: Enthalpy and Entropy

- The text reviews the first and second laws of thermodynamics, establishing that energy conservation ($\Delta E = q + w$) and the concept of entropy (S) are crucial for understanding reaction spontaneity.
- Enthalpy (ΔH) is discussed in the context of exothermic ($\Delta H < 0$) and endothermic ($\Delta H > 0$) reactions, while entropy changes (ΔS) capture the degree of dispersal or "spread" of energy and matter.
- The Gibbs free energy equation, $\Delta G = \Delta H - T\Delta S$, is derived and emphasized as a critical tool for predicting whether a reaction will proceed spontaneously.

Integration and Application

- The chapter culminates in the integration of these concepts by showing how both the energetic (enthalpy) and dispersive (entropy) factors combine to determine the overall free energy change of a system.
- Examples like the evaporation of water and the reaction between $Ba(OH)_2 \cdot 8H_2O$ and NH_4SCN are used to illustrate that even endothermic processes can be spontaneous when accompanied by significant increases in entropy.

In summary, this chapter provides a comprehensive framework for understanding the energetics of biochemical reactions. It equips students with the tools to analyze how free energy, equilibrium, and the interplay between enthalpy and entropy govern the direction and extent of reactions—knowledge that is essential for exploring metabolism and other complex cellular processes.

1.4: Genetic Foundations

Learning Goals (ChatGPT o3-mini)

1. **Evolution and Natural Selection**
   - Describe the theory of biological evolution by natural selection and explain how heritable genetic variation drives adaptation over generations.
   - Differentiate between genotype and phenotype, and discuss the role of allelic variation in determining phenotypic traits.
2. **Molecular Basis of Heredity**
   - Explain how the chemical structure and mutability of DNA underpin natural selection and evolutionary change.
   - Relate the roles of DNA, RNA, and proteins in transmitting and expressing genetic information.
3. **The Genetic Code and Its Decipherment**
   - Describe the triplet nature of codons and explain how codon specificity determines the amino acid sequence in proteins.
   - Summarize key experiments (e.g., those by Nirenberg, Matthaei, and Khorana) that led to the elucidation of the genetic code.
4. **Central Dogma of Molecular Biology**
   - Outline the processes of DNA replication, transcription, and translation, and explain how they interconnect to express genetic information.
   - Compare the differences in these processes between prokaryotes and eukaryotes, with emphasis on the roles of introns and splicing in eukaryotic gene expression.
5. **Mutations and Protein Evolution**
   - Identify different types of mutations (e.g., missense, nonsense, frameshift) and discuss their potential effects on protein function and phenotype.
   - Explain the concepts of homologs (orthologs and paralogs) and analogs, and discuss how protein sequence comparisons can shed light on evolutionary relationships.
6. **Gene Expression and Regulation**
   - Discuss how gene expression is regulated at multiple levels, including transcriptional control and epigenetic modifications, and explain the impact on phenotype and evolution.
   - Evaluate examples of how environmental factors can induce heritable changes in gene expression without altering the DNA sequence.
7. **Modern Molecular Biology Techniques**
   - Explain the principles behind key DNA manipulation techniques such as restriction enzyme digestion, DNA cloning, PCR, and site-specific mutagenesis.
   - Describe the methodologies used in DNA sequencing (e.g., Sanger sequencing and real-time methods) and DNA fingerprinting, and discuss how these techniques have revolutionized biological research and forensic science.
8. **Bioinformatics and Comparative Genomics**
   - Understand how bioinformatic tools are used to compare DNA and protein sequences, infer phylogenetic relationships, and predict gene function.
   - Discuss the challenges and limitations of sequence-based gene annotation and the importance of experimental validation.

By achieving these goals, students will develop a comprehensive understanding of the molecular principles underlying evolution, gene expression, and modern genetic technologies, thereby equipping them to engage with advanced topics in biochemistry and molecular biology.

1.4.1: Introduction

The development of complex biological organisms on our planet has arisen through the evolutionary mechanism of ***natural selection***. The British naturalist Charles Darwin proposed the theory of biological evolution by natural selection in his book, *'On the Origins of Species'*, which was published in 1859. Darwin defined ***evolution*** as "descent with modification," the idea that species change over time, give rise to new species, and share a common ancestor. The mechanism that Darwin proposed for evolution is ***natural selection***. Because resources are limited in nature, organisms with heritable traits that favor survival and reproduction will tend to leave more offspring than their peers, causing the traits to increase in frequency within a population over generations. Thus, natural selection causes populations to ***adapt***, or increasingly well-suited, to their environments over time. Natural selection depends on the environment and requires existing heritable variation in a group.

Natural selection acts on an organism's ***phenotype*** or physical characteristics. ***Phenotype*** is determined by an organism's genetic makeup (***genotype***) and the environment in which the organism lives. When different organisms in a population possess different versions of a gene for a certain trait, each version is known as an ***allele***. It is primarily this genetic variation that underlies differences in phenotype. Only a single gene governs some traits, but the interactions of many genes influence most traits. A variation in one of the many genes contributing to a trait may have only a small effect on the phenotype; together, these genes can produce a continuum of possible phenotypic values.

For example, interactions between equine coat color genes determine a horse's color. Many colors are possible, but changes in only a few genes produce all variations. *Extension* and *agouti* are particularly well-known genes with dramatic effects. For example, differences in the *agouti gene* can help determine whether a horse is bay or black in coloration, and a change to the *extension gene* can, in turn, make a horse chestnut-colored instead (Figure 1.30). Yet other gene variants are responsible for many other coat color possibilities, including palomino, buckskin, and cremello horses.

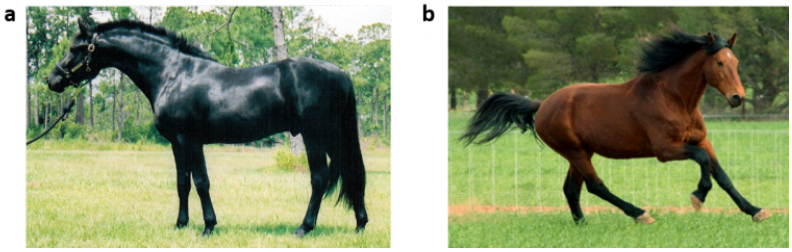


Figure 1.30 Genotype Variations as Determinants of Horse Coat Color. Horses capable of producing the black pigment eumelanin have at least one copy of the dominant *extension gene* (*E/E or E/e*). Interestingly, the *agouti gene* controls the coat's restriction of true black pigment (eumelanin). Horses expressing an *extension dominant gene and* recessive at the *agouti gene locus (a/a*) will be black, as shown in *(a).* Meanwhile, horses dominant for *extension (E/E or E/a)* and dominant for the *agouti genotype* (*A/A* or *A/a)* will never be fully black. Depending on other gene loci, they will instead show coloration patterns such as bay, *as shown in (b). Image (a)* provided by: Serendipityblue; *Image (b)* provided by: CMSporthorses

Thus, the primary molecular mechanism that drives natural selection is controlled by the heritability and mutability of genetic traits housed in the major macromolecule, deoxyribonucleic acid (DNA). In Chapter 4, you will learn about the structural characteristics of DNA. In contrast, Chapter 9 focuses on the biochemical mechanisms involved with DNA replication and also details the importance of the DNA repair process and molecular mechanisms of evolution at the genetic level.

1.4.2: Genetic Code

Notably, the phenotypic traits determined by the genetic makeup of an organism are not controlled directly by the genetic material, DNA, but by the proteins produced from the information housed within the gene. In 1945, geneticist George Beadle proposed the one gene-one enzyme hypothesis, suggesting that genes are highly specific for encoding a protein sequence. However, it would take 16 more years before the biochemical nature of this process was deduced. Efforts to understand how proteins are encoded began after DNA's structure was discovered in 1953. George Gamow postulated that sets of three bases must be employed to encode the 20 standard amino acids living cells use to build proteins, allowing a maximum of $4^3 = 64$ amino acids.

The Crick, Brenner, Barnett, and Watts-Tobin experiment first demonstrated that codons have three DNA bases (Figure 1.31). Marshall Nirenberg and Heinrich J. Matthaei were the first to reveal the nature of a codon in 1961.

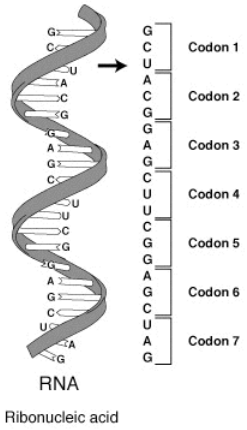


Figure 1.31 Codons Consist of Sets of Three Bases. A series of codons in part of a messenger RNA (mRNA) molecule. Each codon consists of three nucleotides, usually corresponding to a single amino acid. The nucleotides are abbreviated with the letters A, U, G, C. This is mRNA which uses U (uracil). DNA uses T (thymine) instead. This mRNA molecule will instruct a ribosome to synthesize a protein according to this code. Image by Sverdrup

They used a cell-free system to translate a poly-uracil RNA sequence (i.e., UUUUU…) and discovered that the polypeptide they had synthesized consisted only of the amino acid phenylalanine. Thus, they deduced that the codon UUU specified this amino acid.

This was followed by experiments in Severo Ochoa's laboratory that demonstrated that the poly-adenine RNA sequence (AAAAA…) coded for the polypeptide poly-lysine and that the poly-cytosine RNA sequence (CCCCC…) coded for the polypeptide poly-proline. Therefore, the codon AAA specified the amino acid lysine, and the codon CCC specified the amino acid proline. Using various copolymers, most of the remaining codons were determined.

Subsequent work by Har Gobind Khorana identified the rest of the genetic code. Shortly thereafter, Robert W. Holley determined the structure of transfer RNA (tRNA), the adapter molecule that facilitates translating RNA into protein. This work was based upon Ochoa's earlier studies, yielding the latter the Nobel Prize in Physiology or Medicine in 1959 for work on the enzymology of RNA synthesis.

Extending this work, Nirenberg and Philip Leder revealed the code's triplet nature and deciphered its codons (Figure 1.32). In these experiments, various combinations of mRNA were passed through a filter containing ribosomes, the components of cells that translate RNA into protein. Unique triplets promoted the binding of specific tRNAs to the ribosome. Leder and Nirenberg determined the sequences of 54 out of 64 codons. Khorana, Holley, and Nirenberg received the 1968 Nobel for their work.

The three stop codons were named by discoverers Richard Epstein and Charles Steinberg. "Amber" was named after their friend Harris Bernstein, whose last name means "amber" in German. The other two stop codons were named "ochre" and "opal" to keep the "color names" theme.

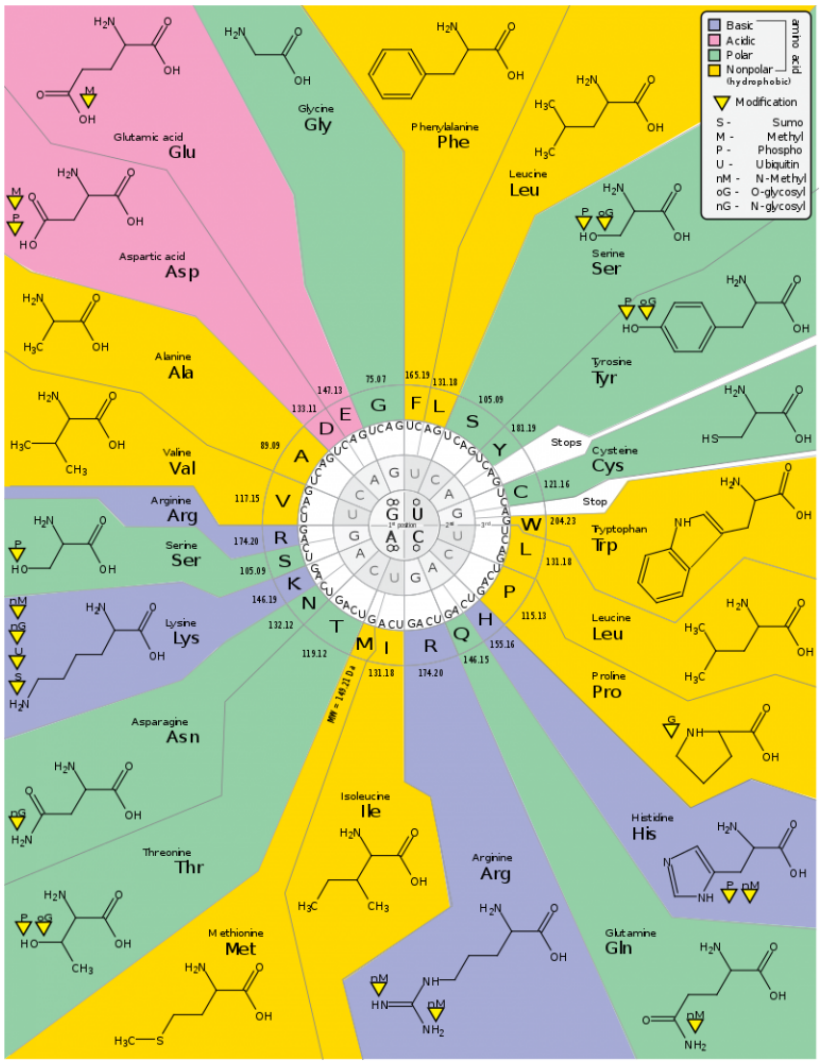


Figure 1.32 The Genetic Code. *Image edited by Seth Miller, Original file designed and produced by: Kosi Gramatikoff courtesy of Abgent*

Each gene contains a reading frame defined by the initial triplet of nucleotides from which translation starts. It sets the frame for a run of successive, non-overlapping codons, known as an ***open reading frame (ORF)***. For example, the string 5′-AAATGAACG-3′, if read from the first position, contains the codons AAA, TGA, and ACG; if read from the second position, it contains the codons AAT and GAA; and if read from the third position, it contains the codons ATG and AAC. Every sequence can, thus, be read in its 5′ → 3′ direction in three reading frames, each producing a possibly distinct amino acid sequence: in the given example, Lys (K)-Trp (W)-Thr (T), Asn (N)-Glu (E), or Met (M)-Asn (N), respectively. When DNA is double-stranded, six possible reading frames are defined: three in the forward orientation on one strand and three in reverse on the opposite strand. A start codon defines protein-coding frames, usually the first AUG (ATG) codon in the RNA (DNA) sequence.

There are three stop codons to terminate the translation process: UAG is *amber*, UGA is *opal* (sometimes also called *umber*), and UAA is *ochre*. Stop codons are also called “termination” or “nonsense” codons. They signal the release of the nascent polypeptide from the ribosome.

Mutations

During the process of DNA replication, errors occasionally occur in the polymerization of the second strand. These errors, called mutations, can affect an organism’s phenotype, especially if they occur within a gene's protein-coding sequence. Error rates are typically 1 error in every 10–100 million bases due to DNA polymerases' “proofreading” ability.

Missense mutations and ***nonsense mutations*** are point mutations that can cause genetic diseases such as sickle-cell disease and thalassemia, respectively. Clinically important ***missense mutations*** generally change the properties of the coded amino

acid residue among basic, acidic, polar, or non-polar states, whereas ***nonsense mutations*** result in a stop codon.

Mutations that disrupt the reading frame sequence by **indels** (insertions or deletions) of a non-multiple of 3 nucleotide bases are known as ***frameshift mutations***. These mutations usually result in a completely different translation than the original RNA and likely cause a stop codon to be read, which truncates the protein. These mutations may impair the protein's function and are thus rare in *in vivo* protein-coding sequences. One reason inheritance of frameshift mutations is rare is that if the protein being translated is essential for growth under the organism's selective pressures, the absence of a functional protein may cause death before the organism becomes viable. Frameshift mutations may result in severe genetic diseases such as Tay–Sachs disease.

Although most mutations that change protein sequences are harmful or neutral, some have benefits. These mutations may enable the mutant organism to withstand particular environmental stresses better than wild-type organisms or reproduce more quickly. In these cases, a mutation will become more common in a population through natural selection. Different sequence variations of the same gene or protein within a single organism, within a population, or between different species are known as ***sequence polymorphisms***. Larger-scale gene duplication events can also lead to evolutionary events.

1.4.3: Similar Proteins

The evolution of proteins is studied by comparing the sequences and structures of proteins from many organisms representing distinct evolutionary clades. If the sequences/structures of two proteins are similar, indicating that the proteins diverged from a common origin, these proteins are called ***homologous proteins***. More specifically, homologous proteins in two distinct species are called ***orthologs***. In contrast, homologous proteins encoded by the genome of a single species are called ***paralogs***. Unrelated genes with separate evolutionary origins, but each encodes proteins with similar functions, are termed ***analogs*** (Figure 1.33).

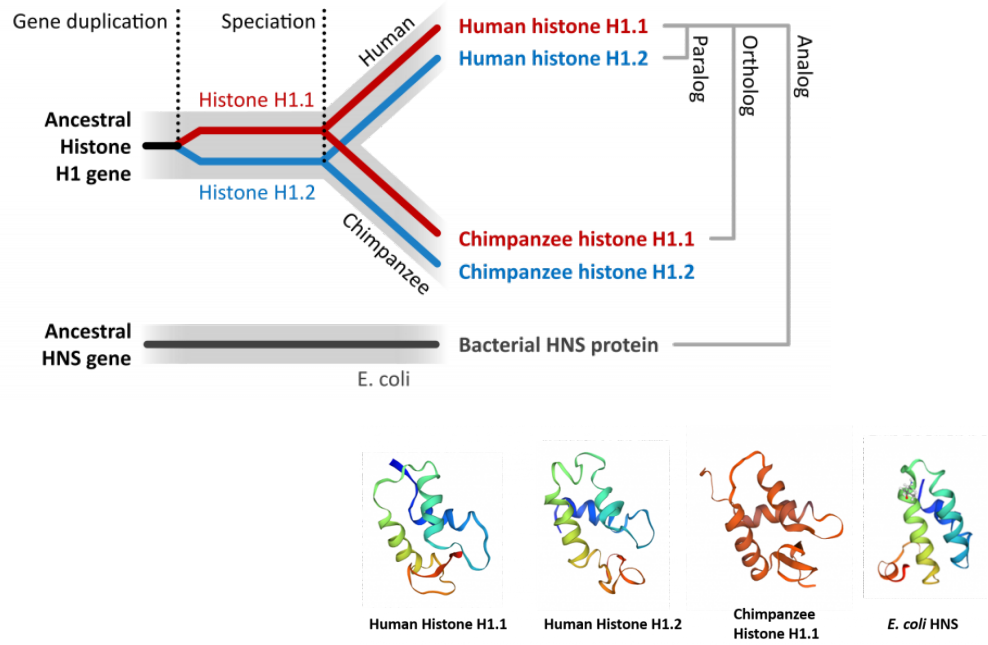


Figure 1.33 Genetic Evolution of Protein Sequences. *(Upper Panel)* An ancestral gene duplicates to produce two paralogs (Gene A and B). A speciation event produces orthologs in the two daughter species. In a separate species, an unrelated gene has a similar function (Gene C) but has a separate evolutionary origin and so is an analog. *(Lower Panel)* 3-D protein models were retrieved or modeled using SWISS-MODEL: Human Histone H1.1 (Q02539), Human Histone H1.2 (P16403), E. coli HNS (P0ACF8). Histone H1.1 from the chimpanzee (*Pan troglodytes* XP_016810512.1) was modeled using Human Histone H1.1 as a template. Note that the *E. coli* HNS protein is typically modeled as a dimer. Only a single monomer is shown here. Upper Image by Thomas Shafee

DNA sequencing techniques have rapidly improved over the last 15 to 20 years, making it possible to sequence the entire genomes of organisms and, thus, predict the entire proteome of an organism based on the translation of the sequenced genome followed by the annotation of predicted ORFs using phylogenetic comparison of similar genes/proteins from other known organisms. This has given rise to the field of ***Bioinformatics,*** which uses computer science, mathematics, and statistical analysis to analyze the large

quantities of biological data created in genome sequencing projects. The phylogenetic relationships, and hence ancestral relationships, of various genes, proteins, and, ultimately, organisms can be established through the statistical analysis of sequence alignments. Such phylogenetic trees have established that the sequence similarities among proteins reflect closely the evolutionary relationships among organisms.

Protein evolution describes the changes in protein shape, function, and composition over time. Through quantitative analysis and experimentation, scientists have strived to understand the rate and causes of protein evolution. Scientists could estimate protein evolution rates by using the amino acid sequences of hemoglobin and cytochrome c from multiple species. What they found was that the rates were not the same among proteins. Each protein has its rate, which is constant across phylogenies (i.e., hemoglobin does not evolve at the same rate as cytochrome c, but hemoglobins from humans, mice, etc. have comparable rates of evolution.). Not all regions within a protein mutate at the same rate; functionally important areas mutate more slowly, and amino acid substitutions involving similar amino acids occur more often than dissimilar substitutions. Overall, the level of polymorphisms in proteins seems to be fairly constant. Several species (including humans, fruit flies, and mice) have similar levels of protein polymorphism.

Gene duplication events followed by mutation can also give rise to paralogs, with unique and different functions within an organism. This can make annotating genomes based on sequence difficult, as homologous protein sequences may not have similar functions *in vivo*. Approximately 10-25% of annotations made on sequence homology are estimated to be incorrect and require experimental validation. For example, human pancreatic ribonuclease is a digestive enzyme that breaks down nucleic acids. The angiogenin protein is a paralog of pancreatic ribonuclease and shares high sequence homology and 3-D shape (Figure 1.34). However, the functions of these proteins are quite different. Angiogenin induces vascularization by activating transcriptional processes in endothelial cells. However, if the function of only one of these homologs were known, it would be easy to mistakenly hypothesize that the homologous protein would be similar in function. Thus, care must be taken when using bioinformatic tools not to overestimate the predictive ability of sequence alignments.

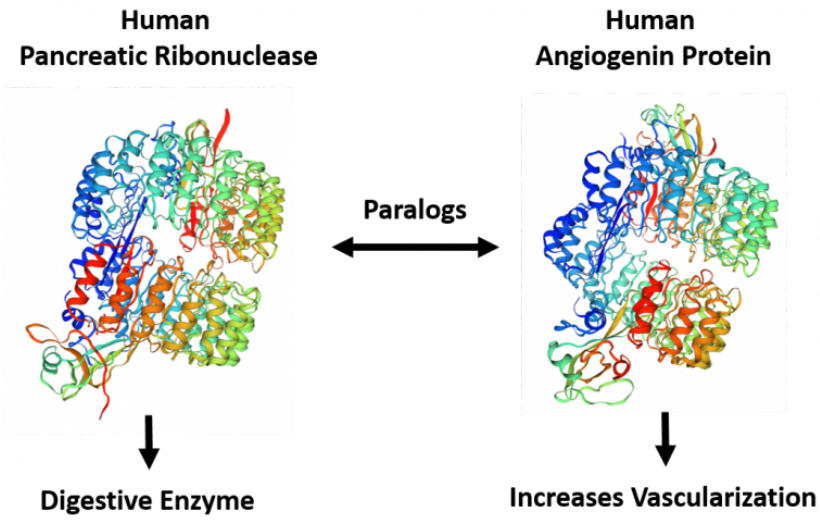


Figure 1.34 Homologous Proteins Do Not Always Have Homologous Functions. In the example above, the digestive enzyme, pancreatic ribonuclease is a paralog of the angiogenin protein and shares an ancestral origin. However, the functions of each of these proteins are quite divergent and have evolved such that they do not share homologous functions. 3-D protein models were retrieved using SWISS-MODEL: Human Pancreatic Ribonuclease (P07998) and Human Angiogenin (P03950)

The control of gene expression is critical in all life processes, allowing for the differentiation of cells to form different body structures and organs, as well as smaller, more reversible changes that allow an organism to respond to different environmental situations and stimuli. In Chapter 12, you will explore the major biochemical mechanisms used to control gene expression within cells. This will include the discussion of a fairly new and exciting field of study known as ***epigenetics***. In addition to the heritability of traits through the passage of genetic information, it is fast becoming clear that the environmental factors that an organism is exposed to throughout its life can affect gene expression without physically altering the DNA sequence and that these changes in expression patterns can be long-lasting and can even be inherited in the following generations. The term ***epigenetics*** means ‘on top of’ or ‘in addition to’ genetics and focuses on the heritable gene expression patterns induced by an organism's exposure or experience within its environment.

For example, in human populations, stressful events such as starvation can have lasting imprints on children who are born under these conditions. These children have higher risks of obesity and metabolic disorders as adults, including the development of type II diabetes. These predispositions can be carried to the children born during starvation and their future children, indicating that environmental events can affect gene expression patterns through multiple generations. In more controlled laboratory experiments using rats, it has been demonstrated that the more a mother rat licks and nurtures its offspring, the calmer and more relaxed the offspring will be as an adult. Mother rats that are less nurturing and ignore their young have offspring that will grow up displaying higher levels of anxiety. These changes are not caused by genetic differences between the offspring but rather by differences in gene expression patterns. Calm and relaxed mice can be altered to show high anxiety by exposing them to agents that alter gene expression patterns. A future chapter will cover mechanisms controlling such heritable alterations in gene expression patterns.

1.4.4: Central Dogma of Biology

DNA encodes the genetic material. It must be replicated during cell division. In transcription, its information is decoded into RNA, which is then decoded to form a protein sequence. Collectively, these processes are referred to as the Central Dogma of Biology. A variant occurs when RNA is decoded into DNA, a process called reverse transcription. These processes are described briefly below and in great depth in subsequent chapters.

Replication

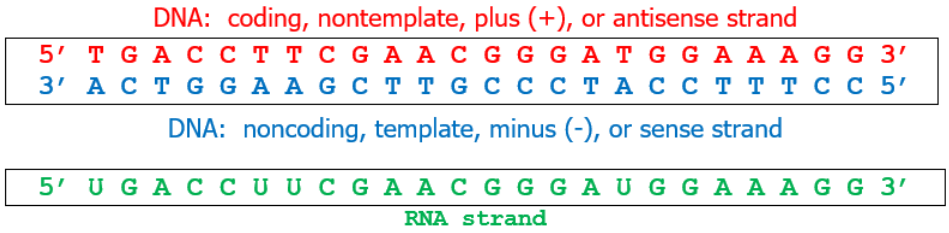
DNA must be duplicated in a process called **replication** before a cell divides. The replication of DNA allows each daughter cell to contain a full complement of chromosomes.

Animation of Replication

Transcription and Splicing

For a given gene, only one strand of the DNA serves as the template for **transcription**. An example is shown below. In this example, the bottom (blue) strand is the template strand, also called the minus (-) strand or the sense strand. It is this strand that serves as a template for mRNA synthesis. The enzyme RNA polymerase synthesizes an mRNA in the 5' to 3' direction complementary to this template strand. The opposite DNA strand (red) is called the coding strand, the nontemplate strand, the plus (+) strand, or the antisense strand.

The easiest way to find the corresponding mRNA sequence (shown in green below) is to read the coding, nontemplate, plus (+), or antisense strand directly in the 5' to 3' direction, substituting U for T.



As we've learned more about the structure of DNA, RNA, and proteins, it become clear that transcription and translation differ in eukaryotes and prokaryotes. Specifically, eukaryotes have intervening sequences of DNA (introns) within a given gene that separate coding fragments of DNA (exons). A primary transcript is made from the DNA, and the introns are sliced out, and exons join in a contiguous stretch to form messenger RNA, which leaves the nucleus. Translation occurs in the cytoplasm. Remember, prokaryotes do not have a nucleus.

- Animation of Transcription
- Animation of mRNA Splicing

Translation

Information in a mRNA sequence is decoded to form a protein. In this process, a triplet of nucleotides (a codon) in the RNA has information about a single amino acid. Translation occurs on a large RNA-protein complex called the ribosome. An intermediary transfer RNA (tRNA) molecule becomes covalently linked to a single amino acid by the enzyme tRNA-acyl synthetase. This "charged" tRNA binds through a complementary anticodon region to the triplet codon in the mRNA. The ribosome/tRNA complex ratchets down the mRNA, allowing a new "charged" tRNA complex to bind at an adjacent site. The two adjacent amino acids form

a peptide bond driven by ATP cleavage. This process repeats until a "stop" codon appears in the mRNA sequence. The genetic code shows the relationship between the triplet mRNA codon and the amino acid that corresponds to it in the growing peptide chain.

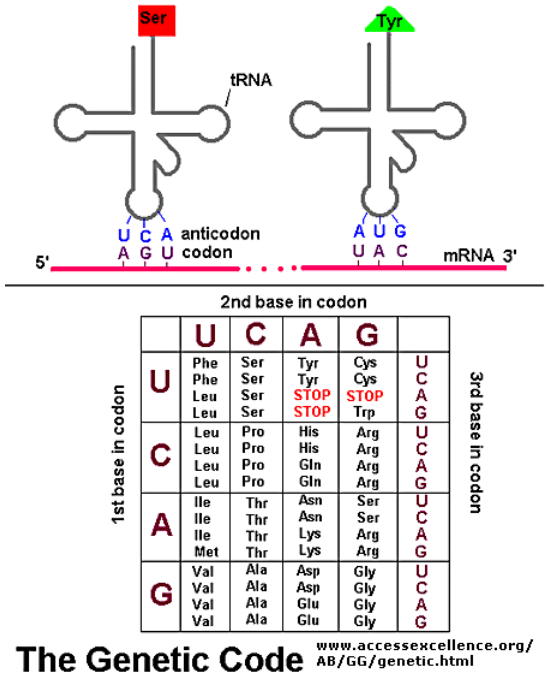


Figure 1.4.1: Codon:Anticodon interactions between mRNA and tRNA. (Copyright; author via source)

As was mentioned in the Protein Chapter (amino acid section), two other amino acids occasionally appear in proteins (excluding amino acids altered through post-translational modification). One is selenocysteine, found in Arachea, eubacteria, and animals. The other is just recently found is pyrrolysine, found on Arachea. These new amino acids are derived from modifications of Ser-tRNA and probably Lys-tRNA after the tRNA is charged with the normal amino acid, which produces selenocys-tRNA and pyrrolys-tRNA, respectively. The pyrrolysine-tRNA recognizes the mRNA codon UAG, which is usually a stop codon, while selenocys-tRNA recognizes UGA, also a stop codon. This usual tRNA complex would recognize only a small fraction of stop codons in mRNA sequences. What determines that recognition is unclear.

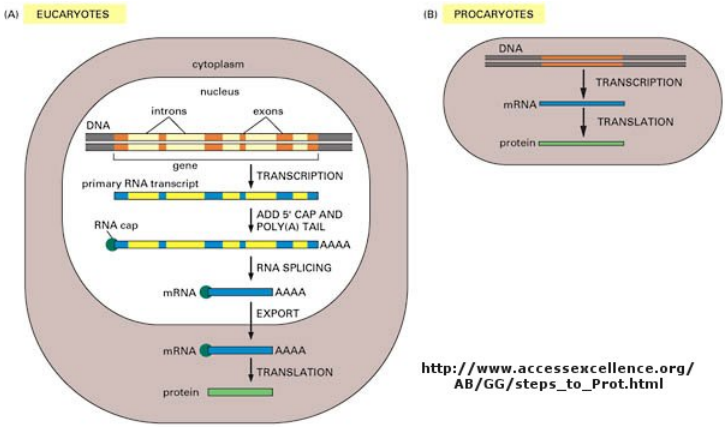


Figure: Central Dogma Differences in Eukaryotes and Prokaryotes

Animation of Translation

1.4.5: What is a gene?

The definition of a gene can differ depending on whom you ask. The word gene has become a cultural icon of our day. Can our genes explain what it is to be human? The definition of a gene has changed with time. Eukaryotic genes contain exons (coding regions) and introns (intervening sequences) that are transcribed to produce a primary transcript. In a post-transcriptional process,

introns are spliced out by the spliceosome to produce a messenger RNA, mRNA, translated into a protein sequence. (See diagram above).

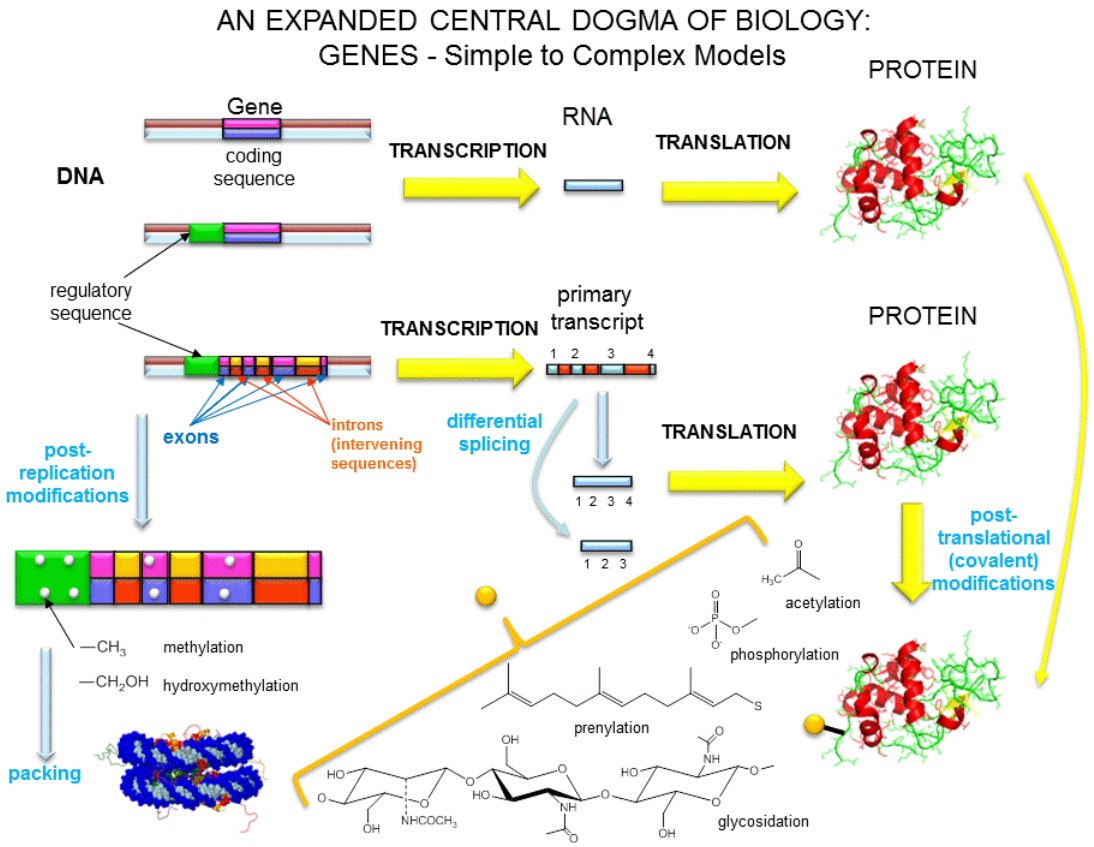


Figure 1.4.1: Figure: A view of genes and their products: Simplicity to Complexity

Over the last 100 years, as our understanding of biochemistry has increased, the definition of a gene has evolved from

- the basis of inheritable traits
- certain regions of chromosomes
- a segment of a chromosome that produces one enzyme
- a segment of a chromosome that produces one protein
- a segment of a chromosome that produces a functional product

The last definition was necessary since some gene products that have functions (structural and catalytic) are RNA molecules. The last definition also includes regulatory regions of the chromosome involved in transcriptional control. Snyder and Gerstein have developed five criteria that can be used in gene identification, which is important as the complete genomes of organisms are analyzed for genes.

1. identification of an open reading frame (ORF) - this would include a series of codons bounded by start and stop codons. This gets progressively harder if the gene has many exons embedded in long introns.
2. specific DNA features within genes - these would include a bias towards certain codons found in genes or splice sites (to remove intron RNA)
3. comparing putative gene sequences for homology with known genes from different organisms but avoiding sequences that might be conserved regulatory regions.
4. identification of RNA transcripts or expressed protein (which does not require DNA sequence analysis as the top three steps do) -
5. inactivating (chemically or through specific mutagenesis) a gene product (RNA or protein).

New findings make it even more complicated to define a gene, especially if the transcripts of a "gene region" are studied. Cheng et al studied all transcripts from 10 different human chromosomes and 8 different cell lines. They found a large number of different transcripts, many of which overlapped. Splicing often occurs between nonadjacent introns. Transcripts were found from both

strands and were from regions containing introns and exons. Other studies found up to 5% of transcripts continued through the end of "gene" into other genes. 63% of the entire mouse genome, comprised of only 2% exons, is transcribed.

1.4.6: The Language of DNA

In this short chapter, you will briefly learn how modern molecular biologists manipulate DNA, the blueprint for all of life. The details will be found in subsequent chapters. The four-letter alphabet (A, G, C, and T) that makes up DNA represents a language that, when transcribed and translated, leads to the myriad of proteins that make us who we are as a species and as individuals. Let's continue with the metaphor that DNA is a language. To master that language, as with any other language, we need to be able to read, write, copy, and edit that language. If you were using a word processor to find one line in a hundred-page document or one article from one book out of the Library of Congress, you would also need a way to search the large print base available. You might want to compare two different copies of files to see if they differ. From the lab and this online discussion and problem set, you will learn how modern scientists read, write, copy, edit, search, and compare the language of the genome. These abilities, acquired over the last twenty years, have revolutionized our understanding of life and given us the potential to alter life for good or evil.

DNA in human chromosomes exists as one long double-stranded molecule. It is too long to study and manipulate in the lab physically. Using a battery of enzymes, the DNA of chromosomes can be chemically cleaved into smaller fragments, which are more readily manipulable. (Similar techniques are used to sequence proteins, which require overlapping polypeptide fragments to be made.) After the fragments have been made, they must be separated from each other to study them. DNA fragments can be separated based on some structural feature that differentiates the fragments from each other. Polarity can not be used since all DNA fragments have negatively charged phosphates in the sugar-phosphate backbone of the molecule. Although each fragment would have a unique sequence, it would be hard to separate all the different fragments by, for instance, attaching some molecule that binds to a unique sequence in the major groove of a given fragment to a big bead and using that bead to separate that one unique fragment. You would need a different bead for each unique fragment! The best way to separate the fragments from each other is to base the separation on the size of the fragment by using electrophoresis on an agarose or polyacrylamide gel.

A carbohydrate extract called agarose is made from algae. Water is added to the extract, which is then heated. The carbohydrate extract dissolves in the water to form a viscous solution. The agarose solution is poured into a mold (like warm jello) and is allowed to solidify. A plastic comb with wide teeth was placed in the agarose when it was still liquid. When the agarose is solid, the comb can be removed, leaving little wells in its place. A solution of DNA fragments can be placed in the wells. The agarose slab with the sample is covered with a buffer solution, and electrodes are placed at each end of the slab. The negative electrode is placed near the well-end of the agarose slab, while the positive electrode is placed at the other end. If a voltage is applied across the agarose slab, the negatively charged DNA fragments will move through the agarose gel toward the positive electrode. This migration of charged molecules in solution toward an oppositely charged electrode is called electrophoresis. Pretend you are one of the fragments.

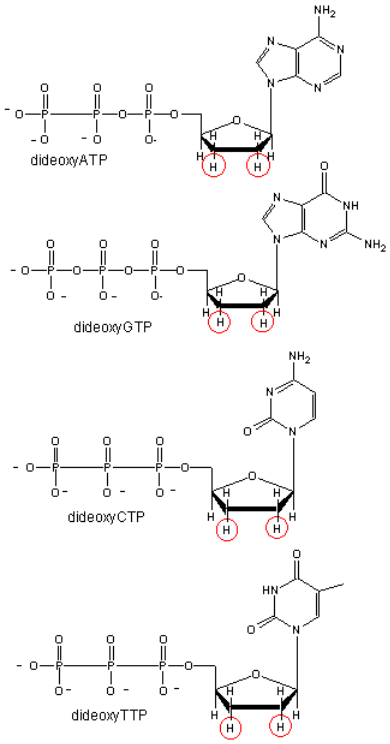
To you, the gel looks like a tangled cobweb. You sneak through the openings in the web as you move straight forward to the positive electrode. The larger the fragment, the slower you move because getting through the tangled web is hard. Conversely, the shorter the fragment, the faster you move. Using this technique and its many modifications, oligonucleotides differing by just one nucleotide can be separated from each other. In the electrophoresis of DNA fragments, a fluorescent, uncharged dye, ethidium bromide, is added to the buffer solution. This dye intercalates -between the base pairs of DNA, which imparts a fluorescent yellow-green color to the DNA when UV light is shown on the agarose gel.

Reading DNA

We will discuss one method of reading the sequence of DNA. This method, developed by Sanger, won him a second Nobel prize. To sequence a single-stranded piece of DNA, the complementary strand is synthesized. Four different reaction mixtures are set up. Each contains all four radioactive deoxynucleotides (dATP, dCTP, dGTP, dTTP) required for the reaction and DNA polymerase. In addition, dideoxyATP (ddATP) is added to one reaction tube. The dATP and ddATP attach randomly to the growing 3' end of the complementary stranded. If ddATP is added, no further nucleotides can be added since its 3' end has an H and not an OH. That's why they call it dideoxy. The new chain is terminated. If dATP is added, the chain will continue growing until another A needs to be added. Hence, a series of discreet fragments of DNA chains will be made, all terminated when ddATP is added. The same scenario occurs for the other three tubes, which contain dCTP and ddCTP, dTTP and ddTTP, and dGTP and ddGTP, respectively. All the fragments made in each tube will be placed in separate lanes for electrophoresis, where the fragments will separate by size.

Didexoynucleotides

Figure: Didexoynucleotides



PROBLEM: As shown below, you will pretend to sequence a single-stranded piece of DNA. The enzyme DNA polymerase adds the new nucleotides to the primer, GACT, in the 5' to 3' direction. You will set up 4 reaction tubes, Each tube contains all the dXTP's. In addition, add ddATP to tube 1, ddTTP to tube 2, ddCTP to tube 3, and ddGTP to tube 4. For each separate reaction mixture, determine all the possible sequences made by writing the possible sequences on one of the unfinished complementary sequences below. Cut the completed sequences from the page, determine the size of the polynucleotide sequences made, and place them as they would migrate (based on size) in the appropriate lane of an imaginary gel, which you have drawn on a piece of paper. Lane 1 will contain the nucleotides made in tube 1, etc. Then draw lines under the positions of the cutout nucleotides to represent DNA bands in the gel. Read the sequence of the complementary DNA synthesized. Then write the sequence of the ssDNA that was to be sequenced.

5' T C A A C G A T C T G A 3' (STAND TO SEQUENCE)

3' G A C T 5' (primer)

3' G A C T 5' (primer)

3' G A C T 5' (primer)

3' G A C T 5' (primer)

3' G A C T 5' (primer)

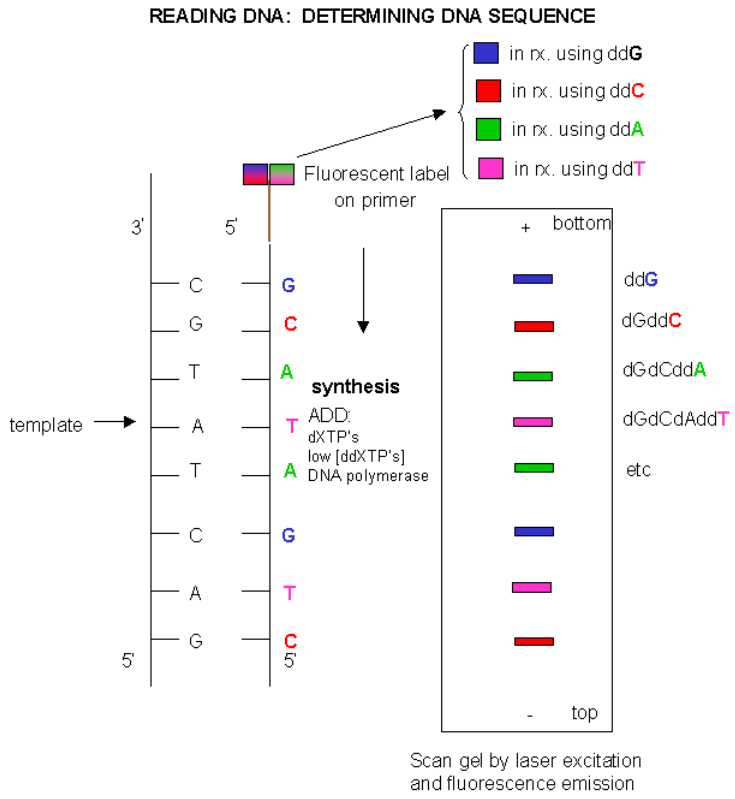
3' G A C T 5' (primer)

3' G A C T 5' (primer)

3' G A C T 5' (primer)

Since the DNA fragments have no detectable color, they can not be directly visualized in the gel. Alternative methods are used. In the one described above, radiolabeled ddXTP's were used. Once the sequencing gel is run, it can be dried, and the bands can be visualized by radioautography (also called autoradiography). An X-ray film is placed over the dried gel in a dark environment. The radiolabeled bands will emit radiation, exposing the X-ray film directly over the bands. The film can be developed to detect the bands. The primer can be labeled with a fluorescent dye using a newer technique. If a different dye is used for each reaction mixture, all the reaction mixtures can be run in one gel lane. (Only one reaction mix containing all the ddXTPs together is performed.) The gel can then be scanned by a laser, which detects fluorescence from the dyes at different wavelengths.

Figure: DNA sequencing using different fluorescent primers for each ddXTP reaction



One recent advance in sequencing allows for real-time determination of a sequence. The four deoxynucleotides are each labeled with a different fluorophore on the 5' phosphate (not the base as above). A tethered DNA polymerase elongates the DNA on a template, releasing the fluorophore into solution (i.e., the fluorophore is not incorporated into the DNA chain). The reaction occurs in a visualization chamber called a zero mode waveguide, a cylindrical metallic chamber with a width of 70 nm and a volume of 20 zeptoliters (20×10^{-21} L). It sits on a glass support through which laser illumination of the sample is achieved. Given the small volume, non-incorporated fluorescently tagged deoxynucleotides diffuse in and out in the microsecond timescale. When a deoxynucleotide is incorporated into the DNA, its residence time is in the millisecond time scale. This allows for prolonged detection of fluorescence, which results in a high signal-to-noise ratio. Newer technology in which sequence is done by moving DNA through pores in membranes could bring sequencing down to $1000/genome or less.

- Animation of Sanger Sequencing
- Nanopore sequencing

Writing DNA

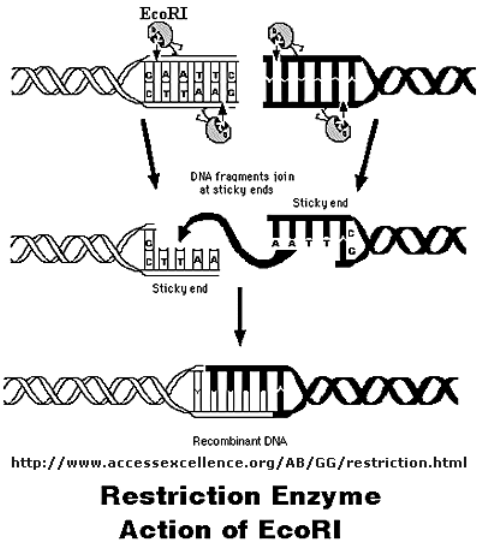
Oligonucleotides can be synthesized on a solid bead. Adding one nucleotide at a time can control the sequence and length of the oligonucleotide.

Copying DNA

Several methods exist for copying a sequence of DNA millions of times. Most methods use plasmids (found in bacteria) and viruses (which can infect any cell). The DNA of the plasmid or virus is engineered to contain a copy of a specific DNA sequence of interest. The plasmid or virus is then reintroduced into the cell, where amplification occurs.

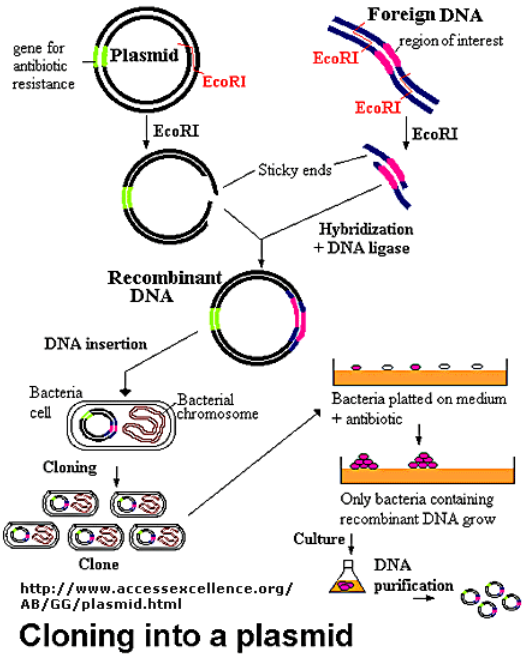
Initially, DNA containing a gene or regulatory sequence of interest is cut at specific places with an enzyme called a restriction endonuclease, or restriction enzyme for short. The enzyme doesn't cleave DNA anywhere but at "restricted" places in the sequence, much as an endoprotease cleaves a protein after a given amino acid within a protein chain. Instead of cleaving one strand, as in proteins, the restriction endonuclease must cleave both strands of dsDNA. It can cut the strands cleanly to leave blunt ends or, in a staggered fashion, to leave small tails of ssDNA. Multiple such sites exist at random in the genome. The gene of interest must be flanked on either side by such a sequence. The same enzyme is used to cleave the plasmid or virus DNA.

Figure: Cleaving DNA with the Restriction Enzyme EcoR1



The foreign fragment of DNA can then be added to the plasmid or viral DNA to make a recombinant DNA molecule. This technique of DNA cloning is the basis for the entire field of recombinant DNA technology.

Figure: Cloning a Restriction Fragment into a Plasmid



Animation of Gene Splicing

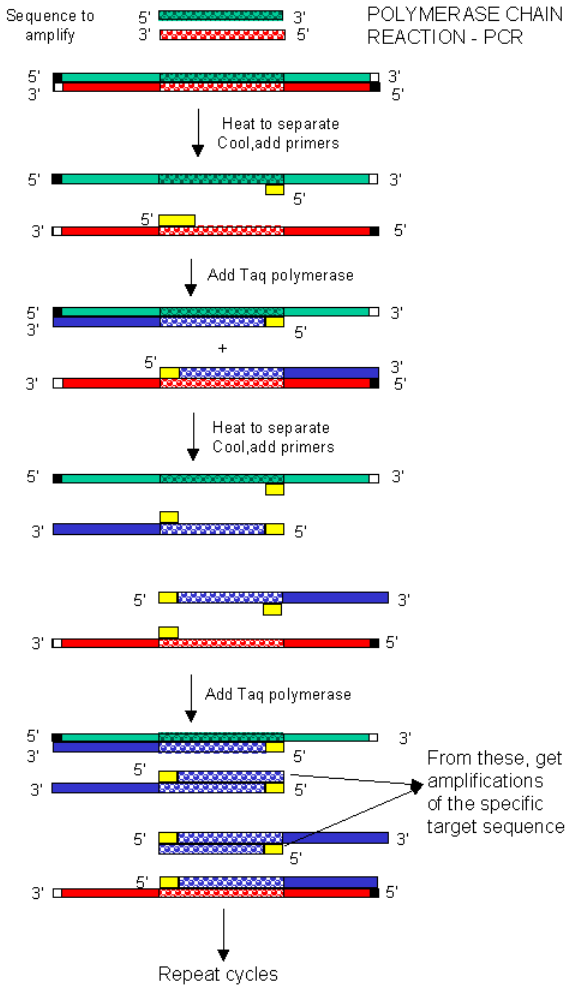
The plasmid can be added to bacteria, which take it up in a process called **transformation**. The plasmid can be replicated in the bacteria, which will copy the DNA fragment of interest. Typically, the plasmid carries a gene that can make the bacteria resistant to an antibiotic. Only bacteria that carry the plasmid (presumably the insert) will grow. To isolate the desired fragment, the plasmids are isolated from bacteria and cleaved with the same restriction enzyme to remove the desired fragment, after which it can be purified. In addition, the bacteria can be induced to express the protein from the foreign gene. In lab 4, we will transform bacteria with a plasmid containing the gene for human adipocyte acid phosphatase beta, HAAP-B, and induce gene expression.

A similar method can be used to copy DNA, in which the foreign fragment is recombined with the DNA of a bacteriophage, a virus that infects bacteria like E. coli. The recombinant DNA can then be packaged into viruses, as shown below. When the virus infects

the bacteria, it instructs the cells to make millions of new viruses, copying the foreign fragment of interest.

Sometimes, "cloning" or copying a fragment of DNA is not what an investigator really wants. For instance, if the genomic DNA comes from a human cell, the gene will contain introns. If you put this DNA into a plasmid or bacteriophage, the introns go with it. Bacteria can replicate this DNA, but often, one wants not just to copy (amplify) the DNA but also transcribe it into RNA and then translate it into protein. Bacteria, however, can not splice out the intron RNA, so mature mRNA can not be made. If one could clone into the bacteria's DNA without the introns, this problem would not exist. One such possible method exists where you start with the actual mRNA for a protein of interest. In this technique, a dsDNA copy is made from a ss-mRNA molecule. Such dsDNA is called cDNA, for complementary or copy DNA. This can then be cloned into a plasmid or bacteriophage vector and amplified as described above.

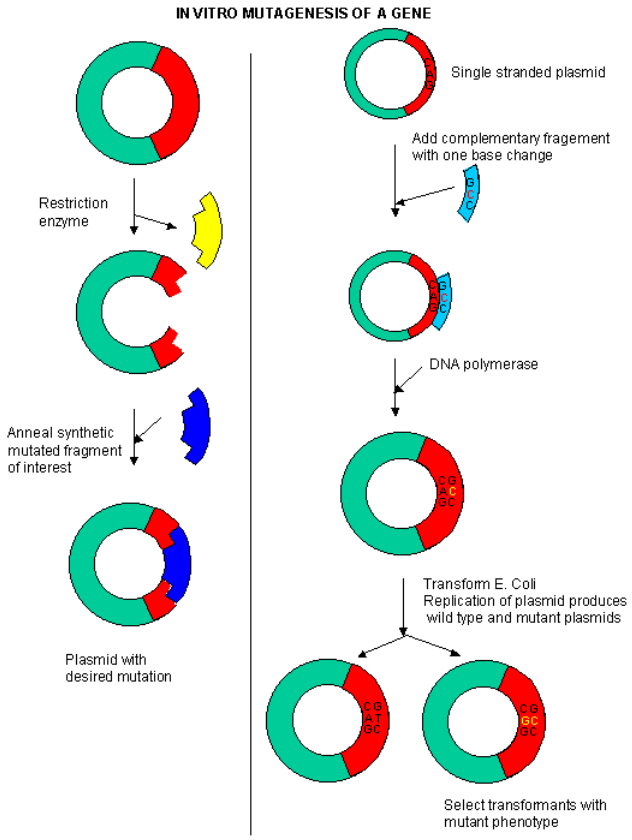
In the mid-'80s, a new method was developed to copy (amplify) DNA in a test tube. It doesn't require a plasmid or a virus. It just requires a DNA fragment and some primers (small oligonucleotides complementary to sections of DNA on each strand and straddling the section of DNA to be amplified). Just add to this mixture dATP, dCTP, dGTP, dTTP, and a heat-stable DNA polymerase from the organism Thermophilus aquaticus (which lives in hot springs), and off you go. The mixture is first heated to a temperature that causes the dsDNA strands to separate. The temperature is lowered, allowing a large stoichiometric excess of the primers to anneal to the ssDNA. The heat-stable Taq polymerase (from Thermophilus aquaticus) polymerizes DNA from the primers. The temperature is raised again, allowing dsDNA strand separation. On cooling, the primers anneal to the original and newly synthesized DNA from the last cycle, and synthesis of DNA occurs again. This cycle is repeated, as shown in the diagram. This chain reaction is called the polymerase chain reaction (PCR). The target DNA synthesized is amplified a million times in 20 cycles or a billion times in 30 cycles, which can be done in a few hours.



- Animation of PCR

Editing DNA

We will spend much time discussing how specific amino acids could be covalently modified to identify the presence of a specific amino acid's presence or to modify the protein's activity. It is routine to use recombinant DNA technology to alter one or more nucleotides, to either change the amino acid or add or delete one or more amino acids. This technique, called site-specific mutagenesis, is used extensively by protein chemists to determine the importance of a given amino acid in a protein's folding, structure, and activity. The techniques are described in the diagram below;



Searching DNA

Where on a chromosome is the gene that codes for a given protein? One way to find the gene is to synthesize a small oligonucleotide "probe," which is complementary to part of the actual DNA sequence of the gene (determined from previous experiments). Attach a fluorescent molecule to the DNA probe. Then, take a cell preparation where the chromosomes can be seen under the microscope. The base is added, which unwinds the double-stranded DNA helix. A fluorescent probe is added that will bind to the chromosome at the site of the gene to which the DNA is complementary. Hybridization is the process whereby a single-stranded nucleotide sequence (the target) binds through H-bonds to another complementary nucleotide sequence (the probe).

What if you don't know the nucleotide sequence of the gene but know the protein's amino acid sequence, as in the example shown below? From the genetic code table, you could predict the sequence of all possible RNA molecules complementary to the DNA in the gene. Since some amino acids have more than one codon, many possible sequences of DNA could code for the protein fragment. The link below shows all corresponding mRNA sequences that could code for a short amino acid sequence. The 20 mer sequences of minimal degeneracy in the nucleotide sequence should be used as genomic probes.

Comparing DNA

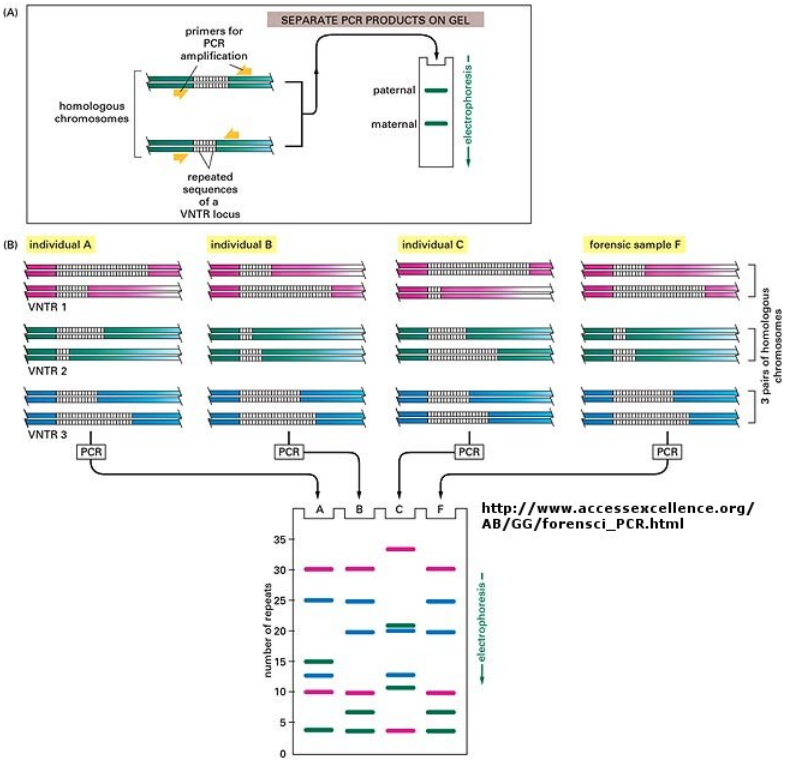
The DNA sequence of each individual must be different from every other individual in the world (except identical twins). The difference must be less than the differences between a human and a chimp, which are 98.5 % identical. Let us say that each of us have DNA sequences that are 99.9 % identical as compared to some "normal humans". Given that we have about 4 billion base pairs of DNA, we are all different in about 0.001 x 4,000,000,000, which is about 4 million base pairs different. This means that, on average, we have one nucleotide difference for each 1000 DNA base pairs. Some of these are in genes, but most are probably in

between DNA, and many are clustered in areas of highly repetitive DNA at the ends of chromosomes (called the telomeres) and in the middle (called the centromeres).

Remember that restriction enzyme sites are also interspersed randomly along the DNA. If some differences in the DNA among individuals occur within the sequences where the DNA is cleaved by restriction enzymes, then in some individuals, a particular enzyme won't cleave at the usual site but at a more distal site. Hence, the size of the restriction enzyme fragments should differ for each person. When cut by a battery of restriction enzymes, each person's DNA should give rise to a unique set of DNA fragments of sizes unique to that individual. Each person's DNA has a unique **Restriction Fragment Length Polymorphism (RFLP).** How could you detect such polymorphism?

You know how to cut sample DNA with restriction enzymes and separate the fragments on an agarose gel. However, an additional step is required since thousands of fragments could appear on the gel, which would be observed as one large continuous smear. If, however, each fragment could be reacted with a set of small, radioactive DNA probes that are complementary to certain highly polymorphic sections of DNA (like telomeric DNA) and then visualized, only a few sets of discrete bands would be observed in the agarose gel. These discrete bands would differ from the DNA bands seen in another individual's gene treated the same way. This technique is called Southern Blotting and works as shown below. DNA fragments are electrophoresed in an agarose gel. The dsDNA fragments are unwound by heating, then a piece of nitrocellulose filter paper is placed on top of the gel. The DNA from the gel transfers to the filter paper. Then, a small radioactive oligonucleotide probe, complementary to a polymorphic site on the DNA, is added to the paper. It binds only to the fragment containing DNA complementary to the probe. The filter paper is dried, and a piece of X-ray film is placed over the sheet. A set of radioactive fragments (not complementary to the probe) are also run. They serve as markers to ensure the gel electrophoresis and transfer to the filter paper occurred correctly.

When this technique is used in forensic cases or paternity cases, it is called DNA fingerprinting. With present techniques, investigators can unequivocally state that the odds of a particular pattern not belonging to a suspect are one million to one. The x-ray film shown below is a copy of real forensic evidence obtained from a rape case. The Southern blot results from suspect 1, suspect 2, the victim, and the forensic evidence are shown. Analyze the data.



1.4.7: Summary

This chapter weaves together fundamental concepts in molecular evolution and modern genetic technologies to provide a comprehensive view of how biological information is stored, transmitted, and modified. It begins by revisiting Darwin's theory of evolution by natural selection, emphasizing that genetic variation—arising from mutations in DNA—is the engine driving the

adaptation and diversification of species. The relationship between genotype and phenotype is explored through examples such as variations in equine coat color, illustrating how specific alleles combine to produce a continuum of observable traits.

The chapter then delves into the molecular basis of heredity, detailing the discovery and decipherment of the genetic code. It highlights key experiments that established the triplet nature of codons and their role in specifying the 20 standard amino acids, thereby forming the basis for protein synthesis. This discovery not only solidified the concept of the Central Dogma—where DNA is replicated, transcribed into RNA, and translated into protein—but also underscored the importance of non-protein-coding sequences and regulatory regions in complex organisms.

Moving forward, the text examines the mechanisms and consequences of mutations, including point mutations (missense and nonsense) and frameshift mutations, and discusses how these genetic alterations can impact protein function and lead to disease. The chapter also distinguishes between homologous proteins (orthologs and paralogs) and analogs, providing insight into protein evolution and the use of sequence comparisons to reconstruct phylogenetic relationships.

A significant portion of the chapter is devoted to modern molecular biology techniques that have revolutionized our ability to read, copy, edit, and compare DNA. Detailed explanations are provided for methods such as restriction enzyme digestion, DNA cloning, and polymerase chain reaction (PCR), along with advanced DNA sequencing techniques—including Sanger sequencing and real-time nanopore sequencing. Additionally, the chapter covers DNA fingerprinting and Southern blotting as powerful tools for genetic analysis and forensic applications.

In summary, this chapter integrates evolutionary theory with molecular mechanisms and technological advances, equipping students with a deep understanding of how genetic information is encoded, maintained, and manipulated—knowledge that is crucial for exploring the frontiers of biochemistry and molecular biology.

1.5: Chapter 1 Questions

Section 1 Questions

Question 1.5.1

In Figure 1.2, two examples of types of enzyme-substrate binding are shown: the Lock-and-Key model and Induced-Fit. What are some situations in which one style of the enzyme would be favored over the other?

Answer

Lock and key enzymes are highly specific for their substrate and therefore do not need a transition state to undergo the catalytic reaction. This could be used for substrate channels like Na+/K+ pumps in which a reaction doesn't need to occur.

Induced fit enzymes utilize a transition state, to convert a substrate into a product. The transition state is able to cause a conformational change in the active site of the enzyme and facilitate high-energy reactions such as breaking or forming chemical bonds

Question 1.5.2

Label the following type of import/export mechanisms as passive, active, or facilitated and explain why: endocytosis, ion channels, pores, transporters/permeases. *Some may have more than one answer.*

Answer

Endocytosis: Active, facilitated. Endocytosis or "cell eating" is a multi-enzyme mediated process that allows the cell to uptake large particles from its environment. This involves membrane modification, protein receptors, and digestive enzymes and organelles working across gradients.

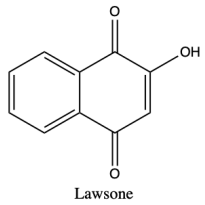
Pores: Passive, facilitated. Once porins establish pores, such as in the nuclear envelope, small molecules like DNA and RNA can passively diffuse in and out of the membrane without the need for carrier proteins.

Ion Channels: Active, facilitated OR passive, facilitated. Active ion channels pump small molecules across a gradient and are typically considered to be "gated," meaning that the enzymes can open and close in a regulated manner to control what is being moved across the membrane. Passive ion channels are permanently open to facilitate transfer and rely on a constantly established concentration gradient to allow for transport to occur.

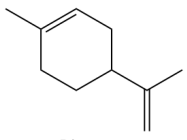
Transporters/Permeases: Active, facilitated. Transporters move larger molecules across a concentration gradient and assist in the movement of soluble proteins and molecules through the hydrophobic membrane

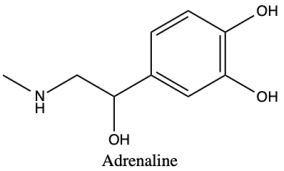
Section 2 Questions

Label the functional groups present in the chemicals shown below:

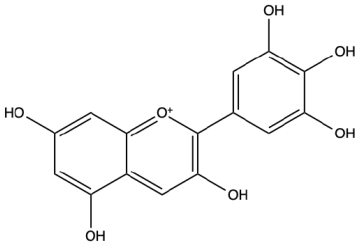


Lawsone

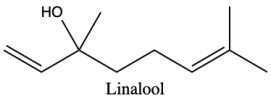

Limonene



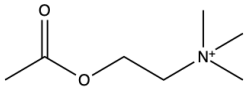
Adrenaline



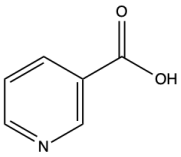
Delphinidin



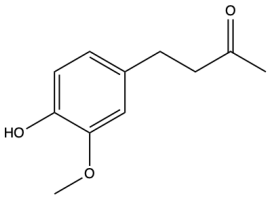
Linalool



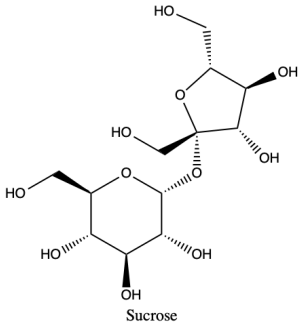
Acetylcholine



Nicotinic Acid



Zingerone



Sucrose

Answers:

Section 3 Questions

1) a. Consider a subset of reactions of glycolysis given below. ΔG'°, substrates, and products are given from colon cancer cells (nmol/g tissue). After examining the conditions of the cell for each enzymatic reaction, predict if the ΔG of the reaction will increase or decrease. (Data from Hirayama A et al. 2009 *Cancer Research*.) The ratio of NAD+/NADH is 10:1 and the concentrations of the cofactors are ATP (110) and ADP (300).

Reaction	ΔG'°	[Substrate]	[Product]
#1 Hexokinase Glucose → Glucose-6-phosphate	-16.6	123	75
#2 Phosphoglucose Isomerase Glucose-6-phosphate → Fructose-6-phosphate	1.67	75	50
#3 Phosphofructokinase Fructose-6-phosphate → Fructose-1,6-bisphosphate	-14.2	50	50
#10 Pyruvate Kinase Phosphoenolpyruvate → Pyruvate	-31.4	5	25
Lactate Dehydrogenase	-25.1	25	25,000

Answer:
Reaction #1 - Increase.
Reaction #2 - Decrease.
Reaction #3 - Increase.
Reaction #10 - Increase.
Lactate Dehydrogenase - Increase.

2) Consider the reaction below along with the thermodynamic properties: ΔH° = -760 kJ/mol, ΔS° = -0.185 kJ/mol K, and ΔG = -705 kJ/mol

$Na^+_{(g)} + Cl^-_{(g)} \rightarrow Na^+_{(aq)} + Cl^-_{(aq)}$

At what temperature would this reaction have an equilibrium constant of 1?

Answer: $\Delta G° = RT\ln(K_{eq})$

Because we want know know the temperature at which $K_{eq} = 1$, and we know that the ln(1) = 0, $\Delta G° = 0$ when $K_{eq} = 1$.

$\Delta G° = \Delta H° - T\Delta S°$
0 = -760 kJ/mol - T(-0.185 kJ/mol K); Rearrange and solve for T = 4108.1 °K

CHAPTER OVERVIEW

2: Water and its Role in Life

Return to Fundamentals of Biochemistry Search Fundamentals of Biochemistry

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2.1: The multiple roles of water

Search Fundamentals of Biochemistry

Learning Goals (ChaptGPT o1, 1/25/25)

- **Interpretation of Duality in Molecular Properties:**
  - Analyze the paradox presented in the riddle (“weak yet strong, strengthens yet destroys”) and relate it to water’s ability to donate and accept protons/electrons.
  - Explain how water's seemingly contradictory behaviors underpin its role in essential biochemical reactions.
- **Water as a Solvent – Thermodynamic Considerations:**
  - Describe how noncovalent interactions (ion-ion, ion-dipole, hydrogen bonding, etc.) govern the solubility of molecules in water.
  - Evaluate the roles of enthalpy and entropy in solvation, including the formation of clathrate structures and the impact of “holes” in the solvent.
- **Solubility and Amphiphilicity:**
  - Investigate how amphiphilic molecules (like fatty acids) self-assemble into micelles, and the biochemical significance of these aggregates in transporting nonpolar molecules in aqueous environments.
  - Discuss experimental approaches, such as liquid/liquid partitioning, to illustrate the principles of solubility and phase separation.
- **Water’s Acid-Base Chemistry:**
  - Examine water’s role as both a Brønsted–Lowry acid and base in chemical reactions, and explain the concept of the leveling effect in aqueous systems.
  - Explore acid-base equilibria involving water, including examples with strong acids (e.g., HCl) and weak acids (e.g., acetic acid).
- **Nucleophilic and Electrophilic Reactions Involving Water:**
  - Analyze water’s dual functionality as a nucleophile and electrophile in various reaction mechanisms, such as ligand substitution in transition metal complexes and electrophilic hydration of alkenes.
  - Compare and contrast the roles of water and hydroxide ions (OH^-) in hydrolysis reactions.
- **Water in Redox Chemistry:**
  - Explore how water acts as an oxidizing or reducing agent in reactions with metals (e.g., Na, Zn) and in biological processes like photosynthesis.
  - Critically assess how water’s redox behavior contributes to generating and controlling reactive oxygen species in biological systems.
- **Molecular Modeling and Visualization:**
  - Utilize interactive molecular models (such as iCn3D) to visualize how water interacts with biomolecules, highlighting the separation of polar and nonpolar regions in structures like micelles and lipoproteins.
  - Interpret structural models of protein complexes (e.g., ferritin, hemoglobin) to understand how water’s chemical properties influence the stabilization and function of macromolecular assemblies.
- **Biochemical Implications of Water’s Unique Properties:**
  - Develop hypotheses on how water’s contrasting properties are essential for life, linking its molecular behavior to its role in processes like nutrient transport, enzyme activity, and cellular structure formation.
  - Critically assess experimental data and current research that highlight the centrality of water in maintaining biochemical homeostasis.

These goals are intended to reinforce theoretical knowledge and encourage critical thinking about how fundamental chemical principles manifest in complex biological systems.

"Nothing in the world is as soft and yielding as it,
Yet nothing can better overcome the hard and strong,
For they can neither control nor do away with it.
The soft overcomes the hard,
The yielding overcomes the strong;"

These words come from Lao Tzu's Tao Te Ching. Let's convert this into a chemical riddle and apply it to biochemistry at the nanoscopic level!

"What it loses, it gains,
What it donates, it accepts,
It is weak yet strong,
It strengthens yet destroys;"

What is it? The answer (one of many possible) is water! It gains and loses protons, donates and accepts electrons, can be both a weaker or stronger acid/base or oxidizing/reducing agent and can lead to crystal formation or dissolution, depending on circumstances. Water, at least on our planet, appears necessary for life. We know of no biological life form that exists without it. This molecule has many unique properties, making it unique compared to most other liquids and optimal for the type of life on Earth. It has contrasting and oppositional properties. Let's investigate a few.

2.1.1: Water as a solvent

Solubility is a property that depends on the nature of both solute and solvent. To a first approximation, we tell students in introductory chemistry and biology courses that for a solute to dissolve in a solvent and form a solution (an example of a homogenous mixture), the sum of noncovalent interactions (intermolecular forces) between solute and solvent must be greater than those among solute molecules and those among solvent molecules.

As students advance in chemistry classes, nuance is added to that general understanding as entropic contributions to solubility must be considered. Entropy is often described as the degree of apparent disorder in the system. Given that description, changes in entropy would appear to favor the soluble state as a solution of the solute in solvent would be more disordered. That simple description must be adjusted to account for the ordered state of solvent (a clathrate) surrounding a solute and of "holes" in the solvent that accommodate larger solute molecules. Enthalpy considerations also must be considered. The description of entropy as a measure of disorder is not precise. Rather, it should be described as a measure of the number of microstates of energy or particles available within a system. An increase in entropy would arise from an increase in the number of such available microstates, which could correlate with an increase in the disorder of a system.

Students might often consider a molecule soluble or insoluble in a given solvent. This notion can be reinforced by simple liquid/liquid partitioning experiments in organic chemistry experiments using two immiscible solvents (for example, water and ether). Yet diethyl ether is partially soluble in water (1 g/100 mL). Nonpolar molecules with no or few bond dipoles are generally considered insoluble. Students would know that acetic acid, a two-carbon molecule, is soluble in water, but how many carbons are necessary for the molecule to become essentially insoluble? Molecules with a single polar group (-OH, CO_2H) and a long alkyl/acyl chain are best described as amphiphilic. Amphiphiles like octanol ($C_8H_{17}OH$) and dodecyl sulfate ($CH_3(CH_2)_{10}CO_2H$) can form multimolecular aggregates called **micelles** even as they exist in as a biphasic system, as shown in the following equilibria:

$$C_8H_{17}OH(liq) \leftrightarrow C_8H_{17}OH(aq) \rightleftharpoons C_8H_{17}OH(micelle).$$

Figure 2.1.1 shows an interactive iCn3D model of a micelle below, which consists of 54 self-associated molecules of dodecylphosphocholine fatty acids. It has an almost "complete" separation of polar (on the surface) and nonpolar atoms (buried).

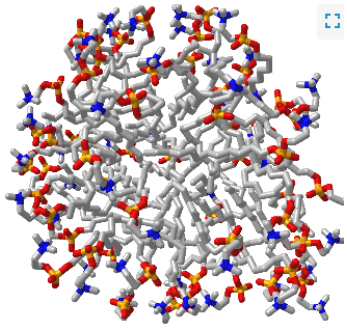


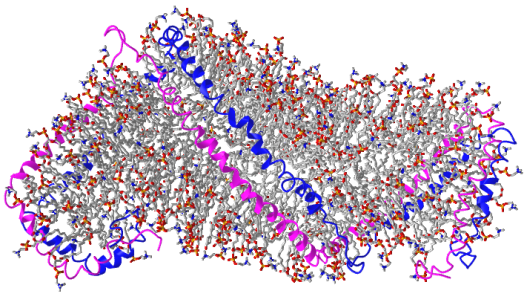
Figure 2.1.1: A Micelle (Copyright; author via source). Click the image for a popup or use this link: https://structure.ncbi.nlm.nih.gov/i...Y7zSYepTQu7yV7.

Note the grey lines representing the nonpolar tails are buried from the surrounding water molecules, which form H bonds with the polar head groups.

Without some limited solubility, the following reaction could not occur:

$$\mathrm{nC_8H_{17}OH(aq) \leftrightarrow 1-C_8H_{17}OH(micelle)}\cdot$$

To solve the general problem of the limited solubility of organic molecules in aqueous-based life, biomolecular structures have evolved to “transport” mostly nonpolar molecules like long-chain carboxylic acids (fatty acids) and cholesterol in circulation. The structure of one such fatty acid and cholesterol-containing particle, nascent high-density lipoprotein (HDL), has been determined by small-angle neutron scattering. Figure 2.1.2 shows an interactive iCn3D model of it. The gray sticks represent the nonpolar acyl tails of the long-chain carboxylic (fatty) acids, while the polar red (oxygen) and blue (nitrogen) atoms surrounding the surface are polar groups connected to the tails. The long magenta and dark blue "helices" represent a protein that wraps around the particle and stabilizes it.



NCBI iCn3D Figure 2.1.2: Nascent HDL (3k2s) (Copyright; author via source). Click the image for a popup or use this external link: https://structure.ncbi.nlm.nih.gov/i...fF85h11SpeYJg6

The same ideas can be applied to the solubility of salts. From introductory chemistry, students will remember general solubility rules (all Gp 1 and Gp 7 salts are soluble). Salts of divalent cations are less soluble as the attractive ion-ion forces within the solid crystal lattice are too strong for the compensatory ion-dipole interactions between the ion and water. Hence, salts of Ca^{2+} and Fe^{2+} ions, such as $CaCO_3$ and $FeCO_3$, are generally insoluble (K_{sp} values of 1.4 x 10^{-8} and 3.1 x 10^{-11}, respectively). Insoluble calcium salts (carbonates and silicates) are needed for shells of Foraminifera and skeletons of vertebrates. Yet free Ca^{2+} and Fe^{2+} ions are found in extracellular and intracellular compartments. Divalent cations like Fe^{2+} can be toxic at a higher concentration, so ways to effectively transport and sequester them have evolved. Figure 2.1.3 shows the structure of human heavy-chain ferritin (4zjk), a

protein that forms a hollow shell in which is stored Fe^{2+} ions (along with counter ions). The model below shows a ferritin with 120 Fe^{2+} ions (spheres) inside the hollow ferritin sphere.

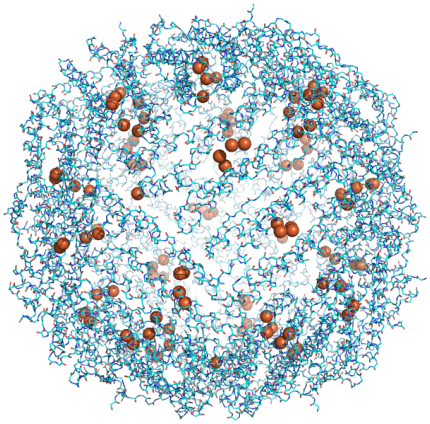


Figure 2.1.3: Human heavy-chain ferritin (4zjk) containing 120 Fe^{2+} ions encapsulated within the hollow sphere formed by the protein.

Finally, let's consider the solubility of gases. The most abundant and relevant ones are O_2 and CO_2, as they are the reactants and products of oxidative respiration. Although the gases contain oxygen atoms, they are nonpolar and have no net dipole. Hence, they are quite insoluble in water. However, they must be soluble enough to allow fish to extract it from water. To solve the solubility problem, evolution has produced proteins like vertebrate hemoglobin that bind oxygen through a transition metal complex containing the Fe^{2+}-heme complex (hemoglobin in vertebrates). Some invertebrates use the transition metal Cu ions in hemocyanins for the same purpose. Figure 2.1.4 shows an interactive iCn3D model of dioxygen (red spheres), bound to a planar heme (yellow highlights) which contains an Fe^{2+} at its center (not shown) at its center in human hemoglobin (6BB5)

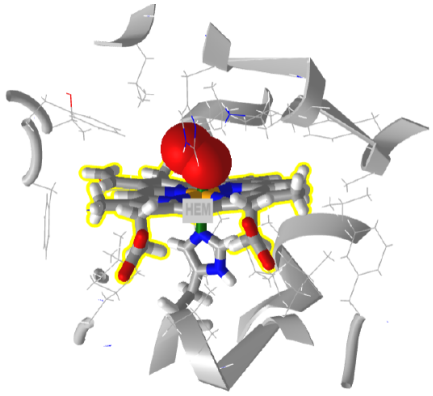


Figure 2.1.4: Oxy-Heme in Human Hemoglobin (6BB5). (Copyright; author via source). Click the image for a popup or use this external link: https://structure.ncbi.nlm.nih.gov/i...DQ5syZ2XTZ4kR9

Water engages in noncovalent interactions with itself and other molecules. Individual noncovalent interactions are weak, but if there are many, they can lead to very strong interactions. You've studied noncovalent interactions before, which may have been described as "intermolecular forces." We prefer the term noncovalent interaction. These include ion-ion, ion-dipole, hydrogen bonds, dipole-dipole, induced dipole-induced dipole, and other variants.

All of these interactions originate in the electrostatic *force* between two charged objects. There is only one law that describes the forces of attraction, and that's Coulomb's Law:

$$F = \frac{kQ_1Q_2}{r^2}$$

From this force, all the electrostatic *interactions* listed above are derived. The magnitude of the attractions for these electrostatic interactions depends on the way charge is distributed in the attracting species. We will explore these in depth in Chapter 2.4.

2.1.2: Water as a reactant: Acids and Bases

H_2O, with its sharable lone pairs and slightly positive Hs, is both a Brønsted–Lowry base and acid. Its acid-base chemistry, hence, is among its most important features.

Water, acting as a base, can react with strong and weak acids. Examples of reactions of a strong acid (HCl) and weak acids (acetic acids and ammonium) with water as a base are shown in Figure 2.1.5.

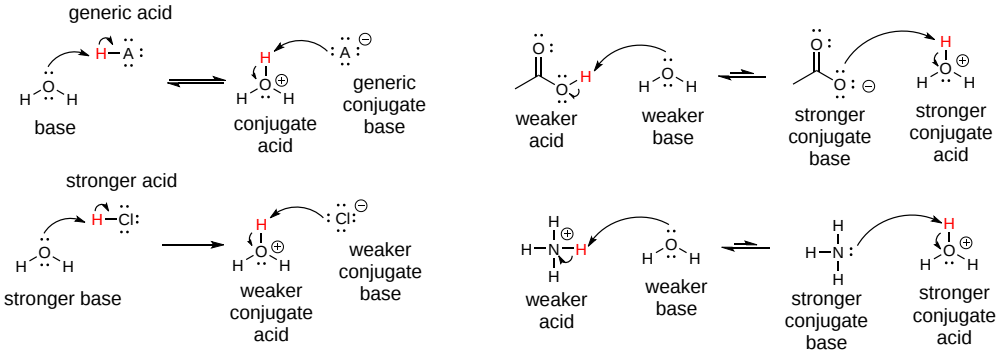


Figure 2.1.5: Reaction of acids with water as a base

Likewise, water can act as an acid as demonstrated in Figure 2.1.6.

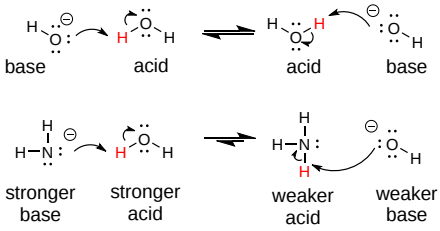


Figure 2.1.6: Reaction of bases with water as an acid

In the first example, no net changes occur. In the second, a negatively charged deprotonated amine (a stronger base than water) can accept a proton from water, which acts as an acid. All acid/base reactions go predominantly in the direction of a stronger acid/strong base to a weaker acid/weaker base. Whether water reacts with a strong acid, such as HCl, or a weak one, like acetic acid, the strongest acid that can exist in an aqueous system is $H_3O^+_{(aq)}$. This is an example of the leveling effect.

2.1.3: Water as a reactant: nucleophile/electrophile

We characterized water as a Brønsted–Lowry acid or base in the reactions above. More generically, we could have said water is a Lewis acid (electron pair acceptor) or Lewis base (electron-pair donor). In many reactions, we can also call water a nucleophile (when it shares its lone pair) or an electrophile (when its slightly positive H atoms react with a nucleophile. Here are some examples.

Reaction of water with a transition metal complex.

This reaction below is effectively a nucleophilic substitution reaction in which water displaces ammonia as a ligand, as shown in Figure 2.1.7 and the following chemical equation.

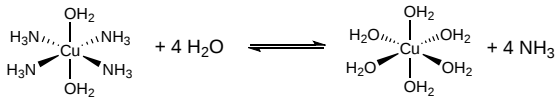


Figure 2.1.7: Reaction of water with transition metal complexes

$$[Cu(NH_3)_4(H_2O)_2]^{2+} + 4\,H_2O \rightleftharpoons [Cu(H_2O)_6]^{2+} + 4\,NH_3$$

Hydration of an alkene

The reaction is catalyzed by adding a proton from an acid (like H_2SO_4), which can be called an electrophilic hydration. Once protonated at the carbon, which makes the most stable carbocation, water, as a nucleophile, attacks the positive carbon to produce the alcohol. These steps are illustrated in Figure 2.1.8.

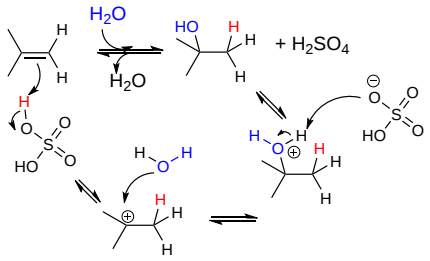


Figure 2.1.8: Mechanism for hydration of an alkene

Nucleophilic substitution at an electrophilic carbonyl

This is a very common reaction. When water is the nucleophile, the reaction is also called hydrolysis. The reactions in Figure 2.1.9 are shown with OH^- as the nucleophile instead of water for simplicity.

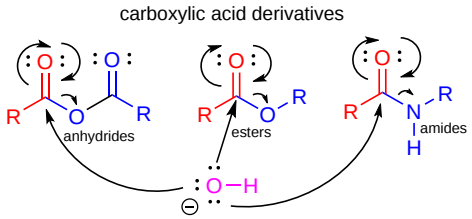


Figure 2.1.9: Reaction of water (OH^-) as a nucleophile

2.1.4: Water as a reactant: Oxidizing/Reducing agent

Everyone knows what happens if you throw a piece of solid Na or K into water. This extremely exothermic reaction releases H_2 gas, which can catch fire and lead to an explosion. The reaction of Na is:

$$2\,Na(s) + H_2O \rightarrow 2\,Na^+(aq) + OH^-(aq) + H_2(g)\cdot$$

The oxidation number of elemental sodium is 0, while Na^+ is +1. This indicates that water oxidized sodium metal and acted as an oxidizing agent.

This reaction occurs with many pure metals, but some that are less reactive (remember the activity series from introductory chemistry?) require acid, a protonated form of water, as shown in the reaction below:

$$Zn(s) + 2\,H_3O^+(aq) \longrightarrow Zn^{2+}(aq) + 2\,H_2O(l) + H_2(g)$$

As in acid/base reactions, in a redox reaction, an oxidizing agent and a reducing agent form a new oxidizing and reducing agent. Other reactants can oxidize water to form oxygen. Consider fluorine gas, for example:

$$3\,F_2 + 2\,H_2O \longrightarrow O_2 + 4\,HF$$

F2 is a stronger oxidizing agent (as you would surmise from its electronegativity) than O2, so the reaction proceeds vigorously to the right.

Of more biological relevance is the oxidation of water to produce O_2 in photosynthesis, a complex series of reactions that is effectively the reverse of combustion:

$$6\,CO_2(g) + 6\,H_2O(l) \rightarrow C_6H_{12}O_6(s) + 6\,O_2(g)\cdot$$

This endergonic reaction requires a large energy input and produces the potent oxidizing agent O_2. The special oxygen-evolving complex in photosynthesis is a powerful oxidant that can oxidize H_2O to form the weaker oxidizing agent O_2.

2.1.5: Summary

Chapter Summary

This chapter delves into water's unique and paradoxical role in biochemistry, using a chemical riddle as an engaging entry point. The riddle—“What it loses, it gains; What it donates, it accepts; It is weak yet strong; It strengthens yet destroys”—is a metaphor

for water's ability to act in multiple, seemingly opposing ways. At the heart of the discussion is the recognition that water's versatile chemical behavior underpins many critical processes essential to life.

Key Concepts Covered:

- **Dual Nature of Water:**
  Water's capacity to donate and accept protons and electrons makes it an ideal medium for countless biochemical reactions. Its ability to behave as a weak or strong acid/base and as an oxidizing or reducing agent illustrates its adaptability in various chemical environments.
- **Water as a Solvent:**
  The chapter emphasizes water's role as a universal solvent in biological systems. It explains how the interplay of noncovalent interactions—such as hydrogen bonds, ion-dipole, and van der Waals forces—facilitates the dissolution of solutes. The discussion extends into the nuanced contributions of enthalpy and entropy, including the formation of structured solvent shells (clathrates) and the accommodation of solute molecules.
- **Solubility, Amphiphilicity, and Micelle Formation:**
  The text explores how solubility is not a binary trait but a spectrum influenced by the nature of both solute and solvent. It introduces amphiphilic molecules and their tendency to form micelles, a key concept for understanding how hydrophobic compounds are transported in aqueous environments. Models of micelles and lipid-protein assemblies like nascent high-density lipoproteins (HDL) illustrate this point vividly.
- **Acid–Base and Nucleophilic/Electrophilic Reactions:**
  Water's dual role as a Brønsted–Lowry acid/base and as a Lewis acid/base is examined through its reactions with both strong and weak acids and its participation in nucleophilic substitutions and electrophilic additions. The leveling effect in aqueous solutions is highlighted as a fundamental principle governing these reactions.
- **Redox Chemistry of Water:**
  The chapter outlines how water acts as an oxidizing and reducing agent in reactions with metals, a particularly important feature in the context of biological redox processes. Examples include the reaction of water with alkali and transition metals and its critical role in photosynthetic water oxidation.
- **Molecular Modeling and Structural Insights:**
  To reinforce theoretical concepts, the chapter utilizes interactive molecular models, such as those of micelles, ferritin, and hemoglobin. These visualizations help illustrate how water's chemical properties affect the structure and function of biomolecules, influencing everything from protein stabilization to the transport of essential ions.

In summary, the chapter comprehensively examines water as more than just a solvent—it is a dynamic participant in the chemical processes that sustain life. The text provides junior and senior biochemistry majors with a deeper understanding of how water's contrasting properties are harnessed in biological systems by integrating principles of thermodynamics, solubility, acid-base chemistry, redox reactions, and molecular interactions.

2.2: Weak Acids and Bases, pH and pKa

Learning Goals (ChaptGPT o1, 1/25/25)

Below is a series of targeted learning goals designed to deepen understanding of acid–base equilibria, titration analyses, and the influence of environmental factors on ionizable groups in biomolecules.

- **Understand Acid–Base Properties of Biomolecules and Water:**
  - Describe how water acts as both a Brønsted–Lowry acid and base, including its autoionization into H_3O^+ and OH^-.
  - Explain how functional groups in biomolecules (e.g., carboxylic acids, phosphoric acids, amines) acquire or lose protons and how this alters their chemical properties.
- **Master the Mathematical Foundations of Acid–Base Equilibria:**
  - Derive and interpret the equilibrium constant expressions for the autoionization of water and for generic acid dissociation reactions.
  - Calculate pH and pKa values using the definitions pH=−log[H3O+]pH = -\log [H_3O^+] and pKa=−logKapK_a = -\log K_a, and understand their physical significance.
- **Apply the Henderson–Hasselbalch Equation:**
  - Use the Henderson–Hasselbalch equation to predict the protonation state of acids in solution under various pH conditions.
  - Interpret titration curves, identify key points such as the inflection point where pH=pKa, and understand the concept of buffering capacity.
- **Analyze Titration Curves and Charge States:**
  - Explain the stepwise protonation/deprotonation processes depicted in titration curves, including the behavior of monoprotic and polyprotic acids.
  - Predict the net charge on biomolecules (e.g., proteins) by applying titration principles to their ionizable functional groups.
- **Explore Polyprotic Acid Behavior:**
  - Define polyprotic acids and explain why each subsequent deprotonation has a higher pKa due to increasing charge repulsion.
  - Analyze complex titration curves for polyprotic acids and discuss the significance of multiple plateaus corresponding to different ionizable groups.
- **Evaluate Environmental Effects on pKa Values:**
  - Discuss how solvent composition (e.g., water vs. ethanol) and the microenvironment (e.g., protein interior vs. surface) influence the pKa of ionizable groups.
  - Use examples such as aspartic acid in different protein contexts to illustrate how nearby charged or hydrophobic groups alter intrinsic acid strength.
- **Connect Acid–Base Chemistry to Biological Function:**
  - Relate the protonation state of biomolecules to their biological functions, such as enzyme activity, ion transport, and protein–protein interactions.
  - Assess how shifts in pH can modulate biomolecular structure and function through changes in charge states.
- **Develop Practical Skills in Quantitative Analysis:**
  - Utilize experimental titration data and interactive models (e.g., spreadsheets, molecular visualizations) to quantitatively determine the pKa values and buffering ranges of various acids and bases.
  - Critically evaluate how titration data supports our understanding of acid–base equilibria in complex biological systems.

These learning goals reinforce theoretical concepts through quantitative reasoning and practical application, ensuring junior and senior biochemistry majors can integrate chemical principles with biological phenomena.

The previous section described the general acid/base properties of water. Many functional groups in both small and large biomolecules act as acids and bases. Common weak acids are carboxylic and phosphoric acid and their derivatives, which become negatively charged after donating a proton. Common weak bases are amines, which become positively charged on protonation. Such charge acquisition changes the properties of the acid or base. A protonated amine is no longer a nucleophile. A deprotonated carboxylic acid can now engage in an ion-ion IMF. The extent of deprotonation depends on the acidity/basicity of the environment. We have to turn to a bit of mathematics to determine that extent.

2.2.1: Reaction of water with self: Autoionization

As shown in the previous section, water can react with itself to produce H_3O^+ and OH^- as illustrated in Figure 2.2.1.

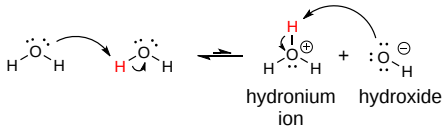


Figure 2.2.1: Reaction of water with self

This autoionization reaction is often represented in a simpler form:

$$\mathrm{H_2O} \rightleftharpoons \mathrm{H^+ + OH^-}.$$

The equilibrium constant for this simplified reaction can be written as

$$K_{eq} = \frac{[H^+]\,[OH^-]}{H_2O} \tag{2.2.1}$$

Given the known value of K_{eq} and the concentration of water (55 M), this can be simply rewritten as

$$K_a = 55K_{eq} = \left[H^+\right]\left[OH^-\right] = 10^{-14} \tag{2.2.2}$$

(see discussion of the pKa of water below.)

Hence pure, neutral water has equal but small concentrations, 10^{-7} M of H_3O^+ and OH^-.

You remember from introductory chemistry and life in general that pure water has a pH of 7. This pH derives from the general formulas for both pH and a new quantity, pKa.

$$pH = -\log\left[H_3O^+\right] = -\log\left(10^{-7}\right) = 7$$
$$pK_a = -\log K_a = -\log\left(10^{-14}\right) = 14$$

Note

Some texts incorrectly use 15.7 for the pKa of water. Here is a link to an explanation of why 14 is better. The wrong value of 15.7 would make the pKa of water higher than that of methanol (15.3), which can't be since the methoxide anion is less stable due to electron release by the methyl group than OH^-.

Generic Bronsted acids with a dissociable proton can be written using the simplified chemical equation below.

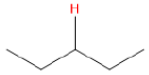
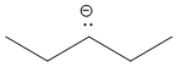
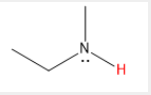
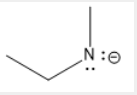
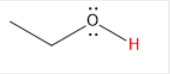
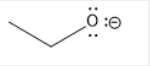
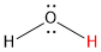
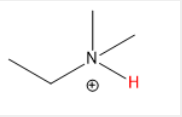
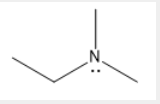
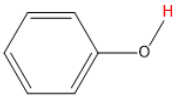
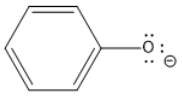
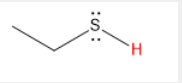
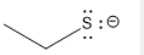
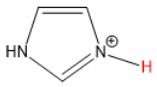
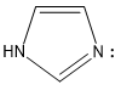
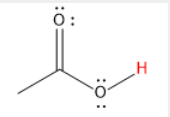
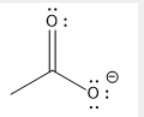
$$\mathrm{HA} \rightleftharpoons \mathrm{H^+ + A^-}$$

The equilibrium constant for this simplified reaction (leaving out water) can be written as

$$\begin{aligned} K_{eq} &= \frac{[H^+]\,[A^-]}{HA} \\ K_a &= [HA]K_{eq} = \left[H^+\right]\left[A^-\right] \\ pK_a &= -\log K_a \end{aligned} \tag{2.2.3}$$

The pKa is a measure of the strength of an acid. The stronger the acid, the larger the Ka and the smaller the pKa.

Here is a table of pK_a values for common acids and functional groups. The pKa values change with different substituents on the acids. The stronger the acid, the weaker the conjugate base. This should make sense as a weak base is unlikely to reabstract a proton and return to its original acidic form. Likewise, the weakest acids produce the strongest conjugate bases, which would reprotonate to return to the weak acid state.

Group	Example	weaker acid	≈ pKa	Conjugate Base	stronger conj. base
alkane			50		
amine			35		
alkyne			25		
alcohol			16		
water			14		
protonated amine			10		
phenol			10		
thiol			10		
imidazole			7		
carboxylic acid			5		
hydrochloric acid			-8		
		stronger acid			weaker conj. base

2.2.2: The Henderson-Hasselbalch Equation

We can find the pKa for small acids in solution in pKa tables. However, from a biochemical perspective, we often need to know the charge state of the acid. Since the pH is approximately constant in organisms (more on that later), we know the $[H_3O^+]$. Hence we can calculate the ratio of A^-/HA using the **Henderson-Hasselbalch equation** (Equation 2.2.5), which is derived below.

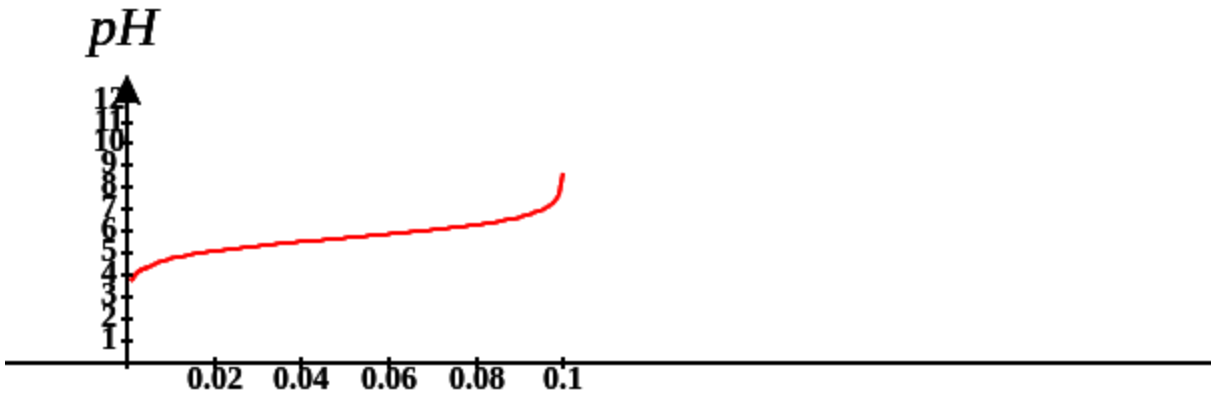
$$\begin{aligned} \mathrm{K_a} &= \frac{\left[\mathrm{H^+}\right]\left[\mathrm{A^-}\right]}{[\mathrm{HA}]} \\ -\log \mathrm{K_a} &= -\log\left[\mathrm{H^+}\right] - \log\left(\left[\mathrm{A^-}\right]/[HA]\right) \\ \mathrm{pK_a} &= \mathrm{pH} - \log\left(\left[\mathrm{A^-}\right]/[\mathrm{HA}]\right) \end{aligned} \tag{2.2.4}$$

which gives the traditional Henderson-Hasselbalch equation below.

$$\mathrm{pH} = \mathrm{pK_a} + \log \frac{\left[\mathrm{A^-}\right]}{[\mathrm{HA}]} \tag{2.2.5}$$

You certainly would have performed titration curve analyses of acids in your chemistry class. What is the chemistry that occurs at each step? Let's assume the pH is low and much lower than the pKa of the acid. From the Henderson-Hasselbalch equation, you would surmise that the ratio of A^-/HA is very small - that is, the acid is essentially fully protonated. That should also make intuitive sense. For a weak acid to be coaxed to give up a proton, a reasonably strong base (like OH^-) should be added. So at low pH, the acid exists just as HA. Now consider adding an amount to NaOH to match the concentration of the ionizable proton. At that point in the titration, the mass balance would suggest that the acid in its protonated state is gone, and all that remains is A^-. What happens if just enough NaOH is added to react with half of the HA? The mass balance would tell us that A^-=HA, and at that point, the pH = pK_a of the acid.

The entire titration curve can be calculated from the Henderson-Hasselbalch equation. A graph of it is shown below.



The graph shifts up as the pKa is increased. The pH starts soaring at the end of the graph after the added hydroxide has reacted with the last ionizable proton. After that, the pH is determined by the concentration of the strong base OH^-. The graph is flattest in the middle of the curve at the inflection point of the curve. Note at this pH, **pH = pKa.** In the middle of the curve, the pH changes least with the addition of small amounts of OH^-. This is the basis of buffering, which will be covered in the next section.

If you know the pH of a solution and the pKa of the ionizable group, you can very quickly estimate the functional group's average charge (protonation) state. Let's see what the Henderson-Hasselbach equation (Equation 2.2.5) predicts under three specific pH states:

pH	log (A^-/HA)	(A^-/HA)	protonation state
2 units < pK_a (more acidic, expect protonated)	-2 = log (A^-/HA)	0.01 = (A^-/HA) = 1/100	fn group about 99% protonated
2 units > pK_a (more basic, expect deprotonated)	2 = log (A^-/HA)	100 = (A^-/HA) = 100/1	fn group about 99% deprotontaed
pH = pKa	0 = log (A^-/HA)	1 = (A^-/HA) = 50/59	fn group 50% protonated

From these simple examples, we have illustrated the ±2 rule to determine the charge state. This rule is used to determine the protonation quickly, and hence the charge state of an acid and its conjugate base, and is extremely important to know and use (and easy to derive). If the potential acid HA is a protonated amine (RNH_3^+), the fully deprotonated state (RNH_2) is uncharged. The fully deprotonated state has a -1 charge if it is a carboxylic acid.

2.2.2.1: Polyprotic Oxyacids

Acids that can donate more than one proton are called **polyprotic acids**. They are typically oxyacids, with the ionizable proton on an oxygen atom, which can form a reasonably stable oxyanion (negative on the oxygen) as the oxygen is electronegative and stabilizes the charge. The negative charge on the conjugate base of oxyacids is further stabilized by resonance delocalization involving the doubly bonded oxygen atom. Figure 2.2.2 shows two of the most biologically relevant oxyacids.

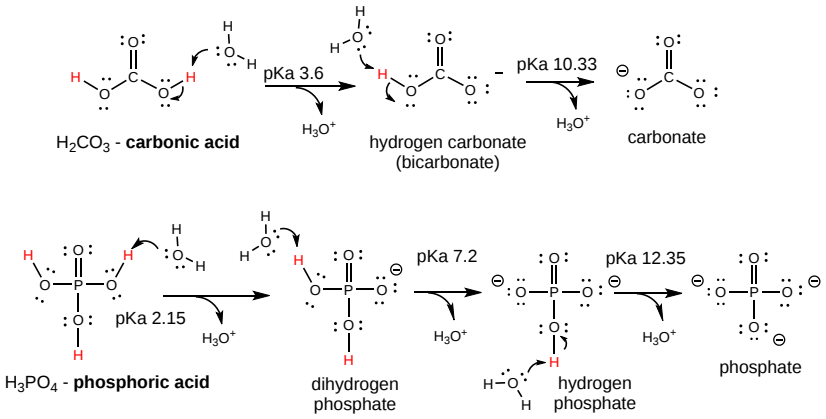


Figure 2.2.2: Reactions of polyprotic acids with water

The pKa for each subsequent ionization is higher since removing a proton from an increasingly more charged molecular ion is more difficult. The titration plot of pH vs NaOH is similar to the graph above but has multiple plateaus at pH=pKa,

Derivatives of phosphoric acid are found in all major classes of biomolecules. Nucleic acids contain a sugar-phosphate link in their backbone. Many proteins become phosphorylated after they are synthesized. Membrane lipids usually contain a phosphate group. A whole class of phospholipids are found in biomembranes.

Titration Curves for Polyprotic Acids

As we will see in a subsequent chapter, all amino acids have an amine and carboxylic acid group, and some have an additional ionizable side chain. Each has its pKa values. Those with three ionizable groups are triprotic acids, much like phosphoric acid. Titration curves for polyprotic acids are more complex than for monoprotic acids. If the pKa values are separated enough, three general plateaus, each centered at the pKa value of the ionizable group, can be seen in their titration curves. If two of the groups are carboxylic acids, no clear plateau will be observed in the region of the titration curve for those groups.

Click on the Excel file link to download a spreadsheet for triprotic acid titrations with sliders to interactively change pKa values, as shown in the image below.

Scroll Bars to change pKa values			
Value	pKa 1	pka 2	pka3
Lower to Higher pKa Value ↓			

2.2.2.2: Charge State of Biomolecules

The Henderson-Hasselbalch equation can be used to determine the charge state of ionizable functional groups (carboxylic and phosphoric acids, amines, imidazoles, guanidino groups) even on a large macromolecule such as proteins, which contain carboxylic acids (weak acids) and amines (weak bases). Figure 2.2.3 shows how the weakly acidic aspartic and glutamic acids, two common amino acids found in proteins, contribute negative charge to the protein and how the amine of the amino acid lysine, a weak base, contributes to positive charge.

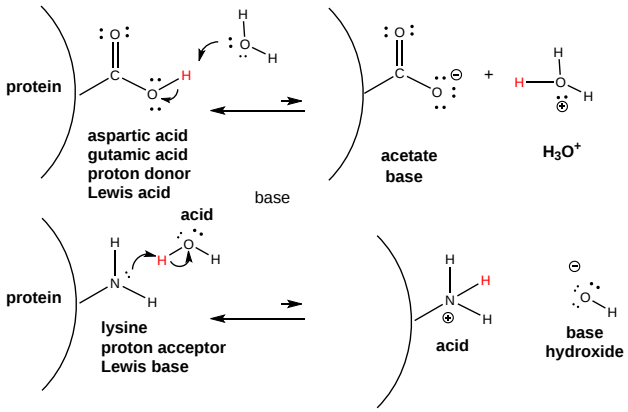


Figure 2.2.3: Deprotonation/Protonation of weak amino acid side chains contributes to protein charge.

Other amino acids that contain an alcoholic function group can also become phosphorylated to produce a phosphoprotein, which converts a neutral ROH group to a phosphoester with a negative two charge, as shown in Figure 2.2.4.

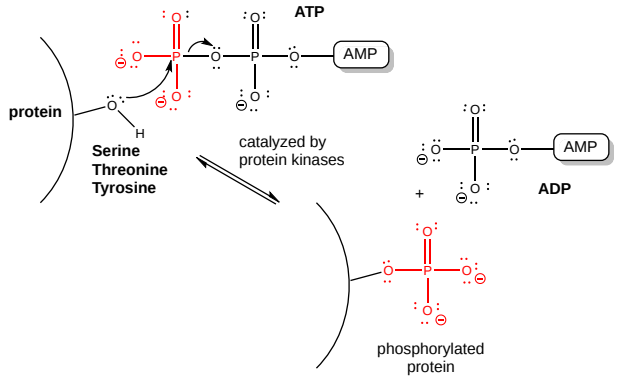


Figure 2.2.4: Phosphorylation of -OH containing side chains of proteins by ATP

2.2.2.3: pKa and Environment

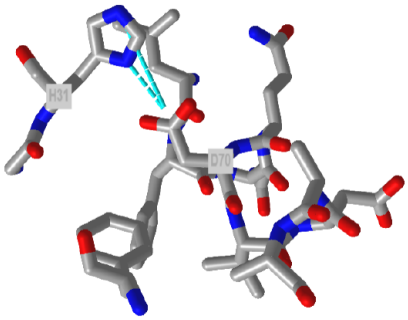
The pKa is a measure of the equilibrium constant for the reaction. And, of course, you remember that ΔG^o = -RT ln Keq. Therefore, pKa is independent of the concentration and depends only on the intrinsic stability of reactants with respect to the products. However, this is true only under a given set of conditions, such as temperature, pressure, and solvent composition.

Consider, for example, acetic acid, which in aqueous solution has a pKa of about 4.7. This weak acid dissociates only slightly to form H^+ (in water the hydronium ion, H_3O^+, is formed) and acetate (Ac^-). These ions are moderately stable in water but reassociate

readily to form the starting product. The pKa of acetic acid in 80% ethanol is 6.87. This can be accounted for by the decrease in stability of the charged products, which are less shielded from each other by the less polar ethanol. Ethanol has a lower dielectric constant than water. The pKa increases to 10.32 in 100% ethanol and a whopping 130 in air!

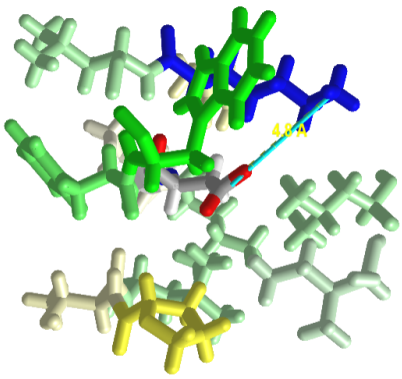
The pKa values of ionizable groups in proteins vary enormously as they depend on the microenvironment of the group. Consider the amino acid aspartic acid (Asp, D), which has a -CH_2CO_2H R-group or "side chain" similar to acetic acid. In a given protein, a given Asp side chain might be on the surface, but another in the same protein might be buried in the protein away from water. You would expect the pKa values for these two Asp side chains to differ. The average pKa for Asp side chains in 78 different proteins was 3.5, less than that of acetic acid (4.7) but not dramatically. However, the range of pKa values for Asp in these proteins was huge, with the lowest being 0.5 (a buried Asp in the protein T4 Lysosome) and the highest being 9.2 in the protein thioredoxin from *E. Coli*.

Figure 2.2.5 shows an interactive iCn3D model of the surrounding environment of Asp 70 (D70) in T4 Lysoszyme. Its pKa has been determined experimentally to be 0.5, which is way stronger than acetic acid. The dotted cyan lines show ion-ion interactions between the -$CH_2CO_2^-$ side chain of Asp 70 (D7) and the positively charged imidazolium group of histidine (H31) in the protein. The distance between the two charged groups is about 3.4 A.



NCBI iCn3D Figure 2.2.5: Surrounding environment of Asp 70 (D70) in T4 Lysoszyme (2b6z). (Copyright; author via source). Click the image for a popup or use this external link: https://structure.ncbi.nlm.nih.gov/i...RRiVn6saN92tU6

The following model shows the surroundings of Asp 26 (D26) in *E. coli* thioredoxin. This particular aspartic acid has a pKa of 9.2. The dark blue group is a surface-exposed positively charged lysine side chain, which can stabilize a negative charge on the Asp 26. Note, however, that it is much farther away than the imidazolium group in T4 lysozyme that stabilizes the negative charge on D70. The rest of the model is colored based on hydrophobicity, which shows that nonpolar groups essentially surround the Asp 26 side chain. These would destabilize a negative charge on the D26, enhancing the stability of protonated (neutral) Asp and elevating its pKa to 9.2.



NCBI iCn3D Figure 2.2.6: Surrounding environment of Asp 26 (D26) in E. Coli thioredoxin (5HR2). (Copyright; author via source). Click the image for a popup or use this external link: https://structure.ncbi.nlm.nih.gov/i...i9BNNdbA2bmP5A

2.2.3: Summary

Chapter Summary

This chapter deepens our understanding of acid–base chemistry as it applies to both water and biomolecules. The discussion begins by reinforcing the fundamental acid–base properties of water, highlighting its autoionization into hydronium (H_3O^+) and hydroxide (OH^-) ions. This sets the stage for quantitatively treating acid–base equilibria, introducing essential concepts such as pH, pKa, and the equilibrium constant (K_a). Students are reminded that the concentration of H_3O determines pH^+, while the pKa value indicates the strength of an acid—the lower the pKa, the stronger the acid.

A major focus is placed on the Henderson–Hasselbalch equation, which provides a practical tool for predicting the ratio of deprotonated to protonated forms of an acid in solution. The chapter illustrates how this equation explains the behavior observed in titration curves, where the point at which pH equals pKa signifies a 50/50 mixture of ionized and unionized species. Through graphical representations and titration analyses, the text clarifies how small pH changes around the pKa minimally affect the pH and buffering capacity of biological systems.

The material expands to cover polyprotic acids—molecules that can donate more than one proton. It explains that each successive proton donation generally occurs at a higher pKa due to the increased difficulty of removing a proton from a more negatively charged species. This section underscores the complexity of titration curves for molecules with multiple ionizable groups, such as phosphoric acid and amino acids, where multiple plateaus may be observed.

Another critical topic is the effect of environmental factors on pKa values. Using examples such as aspartic acid in different protein microenvironments, the chapter demonstrates that the intrinsic pKa of a functional group can shift dramatically depending on local interactions, solvent polarity, and nearby charged residues. These variations are essential for understanding the precise control of protein structure, function, and enzyme activity in vivo.

Overall, the chapter integrates mathematical derivations with biochemical applications, equipping students with the tools to predict biomolecule charge states under various conditions. By connecting acid–base theory to biological function, the text reinforces how pH and protonation dynamics are fundamental to many processes in living systems—from enzyme catalysis to cellular signaling and metabolism.

2.3: Buffering against pH Changes in Biological Systems

Learning Goals

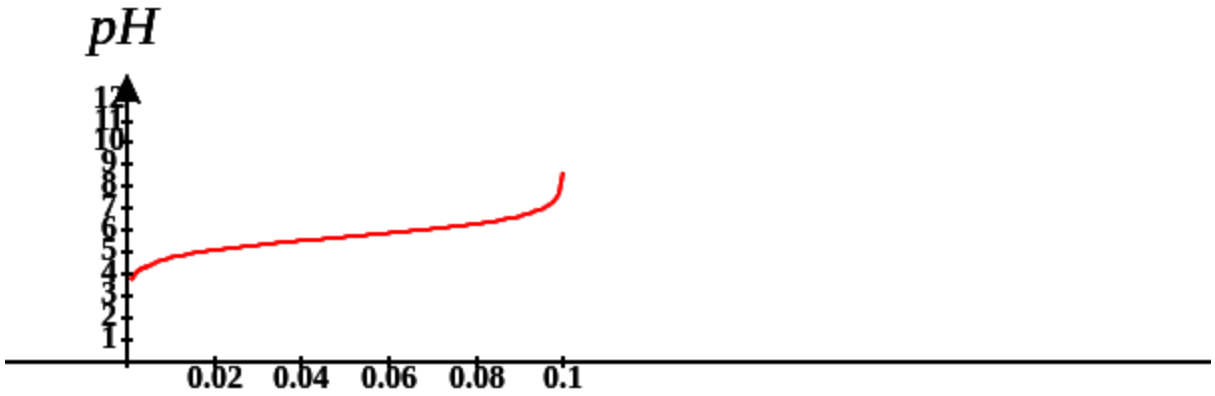
- **Understand pH Homeostasis in Physiology:**
  - Describe how humans maintain blood pH within a narrow range (7.35–7.45) and explain the consequences of deviations (acidosis and alkalosis).
  - Compare pH environments in different cellular compartments (e.g., lysosomes at pH ~4.5 vs. blood).
- **Apply the Henderson–Hasselbalch Equation to Buffer Systems:**
  - Derive and use the Henderson–Hasselbalch equation to predict the ratio of conjugate base to weak acid in buffering systems.
  - Explain why buffering capacity is maximal when pH equals pKa and how small changes in $[A^-]$ or [HA] impact the pH.
- **Analyze the Carbonic Acid/Bicarbonate Buffering System:**
  - Describe the sequential reactions involving CO_2, H_2CO_3, H_3O^+, and HCO_3^- that underlie the carbonic acid/bicarbonate buffering system.
  - Calculate the effective pKa for the system and interpret how the ratio of CO_2 to bicarbonate primes the buffer for rapid response to metabolic changes.
  - Explain how the respiratory system and kidneys work in tandem to regulate this buffer system and, hence, blood pH.
- **Evaluate Other Biological Buffering Agents:**
  - Examine the roles of the phosphate buffering system and protein buffers in maintaining intracellular and extracellular pH.
  - Discuss why certain buffering systems (like the phosphate pair) are less influential in blood compared to the carbonic acid system.
- **Develop Laboratory Buffer Preparation Skills:**
  - Identify strategies for preparing buffered solutions in the lab (mixing separate acid and conjugate base solutions, titrating with strong acid/base, and adjusting volumes).
  - Understand the importance of selecting appropriate buffers (e.g., dihydrogen phosphate/monohydrogen phosphate, HEPES, Tris) based on their pKa and potential interactions (such as binding ions).
- **Link Buffer Systems to Broader Physiological and Environmental Contexts:**
  - Discuss how imbalances in buffering systems can lead to clinical conditions like respiratory acidosis or alkalosis, and explain the physiological responses to such imbalances.
  - Evaluate the impact of increased atmospheric CO_2 on ocean pH and consider how buffering reactions relate to carbon capture strategies in climate change mitigation.

These goals encourage a comprehensive understanding of both the quantitative and qualitative aspects of biological buffers, linking fundamental chemistry with physiological regulation and broader environmental challenges.

2.3.1: Introduction

To ensure homeostasis, humans maintain a pH between 7.35 and 7.45. (Much lower pH values, ≈ 4.5, are found in the lysosome). Lower pH values are associated with metabolic and respiratory acidosis, while higher pH values are characteristic of metabolic and respiratory alkalosis. pH is maintained by buffering systems that consist of a weak acid and base. If you understand the Henderson-Hasselbalch equation from the previous section, buffer systems become easy to understand.

$$\mathrm{pH} = \mathrm{pK_a} + \log \frac{[\mathrm{A^-}]}{[\mathrm{HA}]} \tag{2.3.1}$$



At the curve's inflection point, pH = pKa; at this pH, the system is most resistant to changes in pH when adding either acid or base. At this pH, [HA]=[A^-].

If a bit of a strong acid is added, it would react with the strongest base in the solution, which would be the conjugate base of the weak acid:

$$HCl + A^- \rightarrow HA + Cl^-$$

The reaction goes from a strong acid, HCl, to a weak acid, HA. Its concentration would increase slightly, but it will only ionize to a small extent since it's a weak acid. The [HA] in the Henderson-Hasselbalch equations increases a bit but not enough to change the pH significantly. If the same amount of HCl were added to pure water, it would react completely to form an equal amount of H_3O^+, significantly altering the pH of pure water (7.0).

If a bit of a strong base is added, it will react with the strongest acid in the solution, which would be HA:

$$HA + OH^- \rightarrow H_2O + A^-$$

The reaction goes from a strong base to a weak base A^-. Its concentration would increase slightly but won't affect the pH significantly since it's a weak base. The [A^-] in the Henderson-Hasselbalch equations increases slightly but not enough to change the pH significantly. If the same amount of NaOH were added to pure water, it would react to make the solution basic and significantly alter the pH of pure water (7.0).

To review, buffer solutions contain a weak acid and its conjugate base. They have maximal buffering capacity at a pH = pKa of the weak acid. Generally, a buffered solution can best withstand a change in pH only with $\pm$ 1 pH unit from the pKa.

2.3.2: Biological Buffering Agents

The most relevant biological systems are the carbonic acid/carbonate buffering system, which controls blood and cell pH, and the phosphate buffering system. Proteins with many weak acid and base functional groups can also act as buffering agents.

Carbonic acid/carbonate buffering system: At first glance, the reaction of carbonic acid, H_2CO_3, with water can be written as follows:

$$H_2CO_3(aq) + H_2O(l) \leftrightarrow H_3O^+(aq) + HCO_3^-(aq) \quad pKa = 3.6 \tag{2.3.2}$$

where H_2CO_3 (carbonic acid) is the weak oxyacid, and HCO_3^-(aq) (bicarbonate aka hydrogen carbonate) is its weak conjugate base.

However, this system is more complex since we must add to it another reaction for the formation of H_2CO_3 (aq) in the blood:

$$CO_2(g) \leftrightarrow CO_2(aq) + H_2O(l) \leftrightarrow H_2CO_3(aq) \tag{2.3.3}$$

The [CO_2(aq)] >> [H_2CO_3 (aq)] and their ratio is around 340/1. This makes sense since CO_2 is a very stable molecule. CO_2 in the aqueous form can be readily transported through the blood. Combine the reactions to give the equation below:

$$CO_2(g) \leftrightarrow CO_2(aq) + H_2O(l) \leftrightarrow H_2CO_3(aq) + H_2O(l) \leftrightarrow H_3O^+(aq) + HCO_3^-(aq) \tag{2.3.4}$$

How can carbonic acid with a pKa of 3.6 buffer an aqueous solution at pH 7.5 in the blood and cells? An astute student might have picked up this conundrum. The solution to this problem involves looking at the full set of reactions for the components of the buffer system again. Let's simplify **Equation 2.3.4** since there would be no free "gas bubbles" in blood, so CO_2 (g) = CO_2(aq):

$$CO_2(aq) + H_2O(l) \leftrightarrow H_2CO_3(aq) + H_2O(l) \leftrightarrow H_3O^+(aq) + HCO_3^-(aq) \tag{2.3.5}$$

H_2CO_3 (aq) participates in two different reactions.

Rightwards from H_2CO_3 (aq) :

$$H_2CO_3(aq) + H_2O(l) \leftrightarrow H_3O^+(aq) + HCO_3^-(aq) \tag{2.3.6}$$

Using the simplified equation with H^+ gives

$$K_a = \frac{[H^+]\,[HCO_3^-]}{[H_2CO_3]} \tag{2.3.7}$$

Hence,

$$[H_2CO_3] = \frac{[H^+]\,[HCO_3^-]}{K_a} \tag{2.3.8}$$

Leftwards from H_2CO_3 (aq) :

$$H_2CO_3(aq) \leftrightarrow CO_2(aq) + H_2O(l) \tag{2.3.9}$$

$$K_2 = \frac{[CO_2]}{[H_2CO_3]} \tag{2.3.10}$$

so

$$[H_2CO_3] = \frac{[CO_2]}{K_2} \tag{2.3.11}$$

Since there can be only 1 H_2CO_3 concentration, set Equations 2.3.8 and 2.3.11 equal to each:

$$[H_2CO_3] = \frac{[H^+]\,[HCO_3^-]}{K_a} = \frac{[CO_2]}{K_2} \tag{2.3.12}$$

Solving for [H^+] gives:

$$[H^+] = \frac{[CO_2]\,(K_a)}{[HCO_3^-]\,(K_2)} \tag{2.3.13}$$

Now take the -log of each side to produce an equation similar to the Henderson-Hasselbalch equation.

$$-\log\left[\mathrm{H}^{+}\right] = -\log\left(\frac{\left[\mathrm{CO}_2\right]}{\left[\mathrm{HCO}_3^{-}\right]}\right) - \log\left(\frac{\mathrm{K_a}}{\mathrm{K_2}}\right) \tag{2.3.14}$$

where

$$K_{aEFFECTIVE} = \frac{K_a}{K_2} \tag{2.3.15}$$

This Henderson-Hasselbalch-like equation shows the pH is determined by the K_a/K_2 ratio. $pK_{a\ EFFECTIVE}$ = 6.3. This gives a ratio of CO_2/HCO_3^- of 0.08 = 8/100. There is effectively 12-13 x as much HCO_3^-(aq) as CO_2, making the system primed to react with acid produced metabolically. Yet a second conundrum exists. The pH of the blood (7.4) is outside of the optimal range for a buffer system (in this case, ± 1 pH unit from the pKa, which is 6.3). Again, the system is primed to react with acid as it would move the pH close to the optimal buffering pH of 6.3. Other biological systems also must be involved in maintaining pH.

The respiratory system can quickly adjust pH simply by increasing the exhalation of CO_2. The kidneys can respond more slowly to remove H_3O^+ and retain HCO_3^-. The carbonic acid/bicarbonate buffering system can help us understand how shifting equilibria caused by excessive CO_2 released from rapid, deep breathing or decreased CO_2 release associated with pulmonary disease or shallow rapid breathing can lead to respiratory alkalosis and acidosis, respectively.

- Respiratory alkalosis can be caused by “hyperventilation” or breathing rapidly. This can lead to breathing out (removing) too much CO_2, shifting the above equilibrium to the left, consuming H_3O^+, and increasing pH, making the blood more alkaline. To increase your CO_2 levels, you could breathe into a bag.
- Respiratory acidosis is caused by increased CO_2, which can occur when the lungs aren’t working well and you can’t get rid of the CO2 you produce during respiration. Respiratory acidosis can happen with asthma, pneumonia, lung disease, or anything that decreases respiration rate.

Inhaling CO_2 can lead to panic. This makes sense as it would mimic suffocation, which is lethal to humans. A suffocation response follows. High CO_2 would drive the equilibrium to the right, leading to H_3O^+ production. An acid-sensing ion channel-1a (ASIC1a) in the amygdala, a center of emotion regulation in the brain, has been discovered, and it appears to mediate the panic effect. Panic attacks are sometimes associated with hyperventilation, which leads to alkalosis, not acidosis. Less noted is that when some people panic, they take short, shallow breaths (in a way, almost stopping their breath). This would lead to a buildup of CO_2 since it wouldn’t be released in exhalation. The acid channel in the amygdala would be activated, and a panic response would ensue.

Phosphate buffering system: Phosphates, specifically dihydrogen ($H_2PO_4^-$) and monohydrogen phosphate (HPO_4^{2-}) are also present in the blood. Given the pKa of HPO_4^{2-}, why is PO_4^{3-} **not** present to any significant degree? Since the concentration of phosphates is low in blood, this system is a minor player in blood.

Proteins: Proteins are found in all cellular and extracellular fluids and contain weak acids as buffer components. Proteins contain two amino acids, aspartic acid and glutamic acid) that contain carboxylic acid side chains. Each comprises about 6% of the proteins. In blood, hemoglobin is the most abundant protein by far. Its role in buffering and in O_2 and CO_2 will be discussed in a subsequent chapter.

2.3.3: Making Buffers in the Lab

When studying biomolecules like proteins and nucleic acids in the lab, the pH of the solution is usually maintained under physiological conditions. These macromolecules are either dissolved in or diluted into a buffer solution. Sometimes, it's essential to study their properties and activities as a function of pH. A wide variety of buffer systems have been developed for the lab study of these molecules. The dihydrogen ($H_2PO_4^-$)/monohydrogen phosphate (HPO_4^{2-}) pair is commonly used as the pKa of $H_2PO_4^-$ is 7.21, which makes it physiologically relevant. Care must be taken when selecting buffer systems, as some might bind calcium ions. The pKa of some weak acids varies significantly with temperature as well. Some standard biological buffers are listed below.

Buffers	**pKa (at 25°C)**
MES	6.10
Bis-Tris	6.50
ACES	6.78

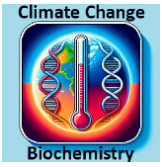
PIPES	6.76
MOPSO	6.90
MOPS	7.20
HEPES	7.48
Tris	8.06
Tricine	8.05
Gly-Gly	8.20
Bicine	8.26
TAPS	8.40
AMPSO	9.00
CAPS	10.40

There are three general ways to make a buffered solution:

1. Make separate and equal concentration solutions for both the weak acid (for example, NaH_2PO_4) and its conjugate base (for example, $Na_2HPO_{4)}$. Use the Henderson-Hasselbalch equation to calculate how much of each should be added to give the correct $[A^-]/[HA]$ ratio (in the case $[HPO_4^{2-}]/[H_2PO_4^{-1}]$) to give the correct pH.
2. Use a pH meter and monitor the pH when adding both solutions together until the desired pH is reached.
3. Make a solution for one of the components (weak acid or its conjugate base) and bring it near its correct volume for the desired molarity. Monitor the pH as you add a concentrated solution of either HCl or NaOH to get the desired pH. Then, bring the solution to the correct volume in a volumetric flask. With this method, you will add counter ions (Cl^- with HCl and Na^+ with NaOH), which you may not want in your buffer solution. Often, it is not a problem.

2.3.4: Climate Change and Carbon Capture

Perhaps humanity's greatest challenge is the effects of global warming and climate change on the biosphere and its health. CO_2 in the atmosphere, $HCO_3^-(aq)$, its soluble form, and CO_3^{2-}, its mainly precipitated form in insoluble carbonate, are key players in the Carbon Cycle and climate change. Follow the link below to see how increasing CO_2 in the atmosphere due to the production and use of fossil fuels affects the pH of the oceans and their health and how CO_2 captured by oceans could be used to decrease atmospheric CO_2 and help stop and reverse global warming.

	32.3: Climate Change - Part 1 - The Carbon Cycle and Carbon Chemistry

2.3.5: Summary

Chapter Summary

This chapter explores the critical role of buffering systems in maintaining pH homeostasis within the human body and in laboratory settings. It begins by discussing how blood pH is tightly regulated between 7.35 and 7.45, with deviations leading to metabolic or respiratory acidosis and alkalosis. The chapter emphasizes that this regulation is achieved through buffer systems—combinations of weak acids and their conjugate bases—that resist significant pH changes upon adding small amounts of strong acids or bases.

A central tool introduced is the Henderson–Hasselbalch equation, which quantitatively relates pH, pKa, and the ratio of conjugate base and weak acid concentrations. The equation explains the buffering capacity at the point where pH equals pKa and underpins the behavior of titration curves and the concept of buffering ranges.

The chapter then focuses on the carbonic acid/bicarbonate buffering system, a primary mechanism for pH regulation in blood and cells. Through a series of interrelated reactions involving CO_2, H_2CO_3, H_3O^+, and HCO_3^-, the system is shown to adjust pH rapidly. The effective pKa of the system, derived from the ratio of CO_2 and bicarbonate, explains how the buffer is primed to neutralize metabolic acids. At the same time, respiratory and renal adjustments fine-tune the pH balance.

Additional biological buffers, including the phosphate system and proteins, are discussed. Although the phosphate buffer plays a minor role in blood due to its low concentration, it is vital in other cellular contexts. Proteins, especially abundant ones like hemoglobin, also contribute to buffering through their ionizable side chains.

Finally, the chapter provides practical insights into buffer preparation in the laboratory. Various strategies for creating buffered solutions are presented, with attention to the selection of buffers based on their pKa values and potential interactions with other ions. The discussion concludes with a broader perspective on how buffer chemistry connects to global issues, such as the role of CO_2 in climate change and carbon capture efforts.

The chapter integrates fundamental chemical principles with biological applications, enabling students to understand and predict how buffer systems stabilize pH in physiological and experimental contexts.

2.4: Solubility in an aqueous world - noncovalent interactions in depth

Learning Goals (ChaptGPT o1, 1/25/25)

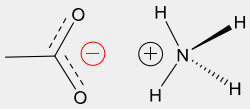
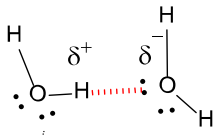
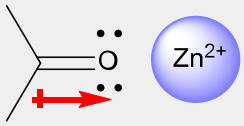
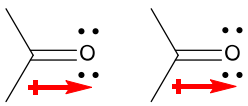
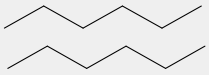
2.4.1: Introduction

In section 2.1, we explored the role of water as a solvent. Using the adage "like dissolves like" that you may have learned in introductory chemistry and biology courses, we can rationalize what substance might dissolve in water. We related this to the types and strengths of attractive interactions between solute and solvent. If, in sum, they are stronger than self-interactions (solute-solute and solvent-solvent), the solute would dissolve (to a reasonable extent) in the solvent. We also discussed entropic contributions to the dissolution process. For now, we will refocus on the noncovalent interactions.

In introductory science courses, noncovalent interactions are often described as intermolecular forces. This term is ambiguous when applied to biochemistry. Take, for example, hydrogen bonds. They occur *between* two water molecules, for example, and *within* larger molecules (like proteins) if hydrogen bond donors and acceptors within the molecule get close enough to each other in space.

The table below summarizes the common noncovalent interactions/"intermolecular forces" you studied in introductory science classes. It is hard enough for students to recognize and identify these interactions between two small molecules let alone in large molecules like proteins. We will explore these in more detail below and give examples of noncovalent interactions *between* small molecules and *within* large ones such as proteins. We'll also add a few more specific examples of interactions.

Noncovalent Interactions - "Intermolecular Forces"

Interaction Type	Example	Distance Dependence	Relative Strength Kcal/mol (kJ/mol)	Direction Dependence
Ion-Ion		1/r	60 (250)	nondirectional
H-Bond			3-15 (12-63)	directional
Ion-dipole		$1/r^2$	3-5 (12-21)	directional
Dipole-dipole		$1/r^3$	0.5-1 (2-4)	directional
Induced Dipole-Induced Dipole		$1/r^6$	0.5 (2) (depend on size)	nondirectional

Although there are many noncovalent interactions, one fundamental principle governs all of them. They all originate in the electrostatic *force* between two charged objects. There is one simple law, Coulomb's Law, which you have discussed in introductory science courses, and one simple equation that describes the electrostatic force:

$$F = \frac{kQ_1Q_2}{r^2} \tag{2.4.1}$$

F is the force (attractive or repulsive) between two particles of charge Q_1 and Q_2 with their centers separated by some distance r. Replace the charges with the masses of two objects, and you will have Newton's Law of Gravitation. Both are inverse squared laws.

All the *interactions* described in the table above arise from the electrostatic force. The magnitude of the attraction depends on how the charge is distributed in the attracting species. Each interaction has different dependencies on distance.

Different words are used to describe noncovalent interactions. This can be distressing to learners who might hear different terms used by chemists and biologists for the same noncovalent interactions. Some use van der Waals forces to describe induced dipole-induced dipole interactions, while others use London dispersion forces or hydrophobic interactions. Others use van der Waals forces to describe *all* noncovalent interactions except ion-ion. To avoid ambiguity, look at the IUPAC Gold Book Compendium of Chemical Terminology, which defines van der Waals forces.

Definition: van der Waals Forces

"The attractive or repulsive forces between molecular entities (or between groups within the same molecular entity) other than those due to bond formation or to the electrostatic interaction of ions or ionic groups with one another or with neutral molecules. The term includes dipole–dipole, dipole-induced dipole, and London (instantaneous induced dipole-induced dipole) forces. The term is sometimes used loosely for the totality of nonspecific attractive or repulsive intermolecular forces". IUPAC. Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). Compiled by A. D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford (1997). Online version (2019-) created by S. J. Chalk. ISBN 0-9678550-9-8. https://doi.org/10.1351/goldbook.

Figure 2.4.1 summarizes covalent and noncovalent interactions, using that definition.

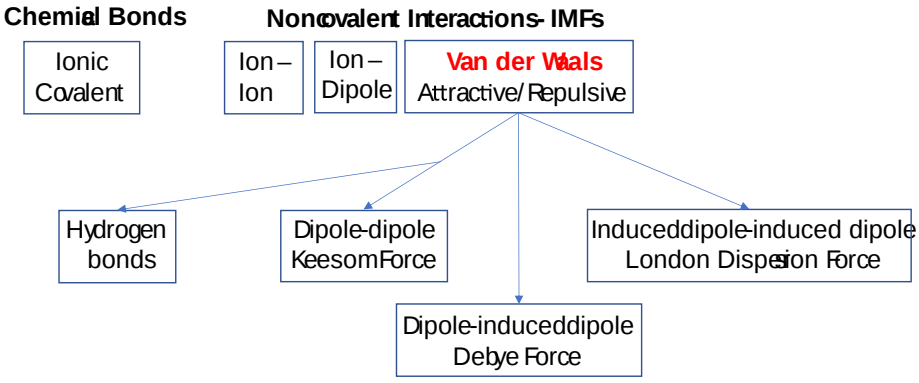


Figure 2.4.1: Noncovalent Interactions

Hydrogen bonds are usually considered a type of dipole-dipole interaction using this definition. Historically, several noncovalent interactions have alternative names based on the person associated with them. Only the names van der Waal and London are commonly used in introductory chemistry courses

Even the word "force" is potentially ambiguous. To a physicist, there are only four known forces:

- gravitational, between two objects with mass
- electromagnetic (between static charges - the electrostatic force, and moving charges - the magnetic force)
- the strong force (holding the nucleus together)
- the weak force (also nuclear and involved in radioactive decay).

We'll try to use the word interaction throughout this book.

Interactions *within* small molecules, such as covalent bonds, and *between* molecules, such as induced dipole-induced dipole, vary as some function of r, the distance between the two interacting particles. Only ion-ion interactions vary as $1/r^2$ ($F= k/r^2$). Attractions lower the overall energy, while repulsions raise it. The system is in its most favored, lowest energy state at some optimal distance.

We just switched from discussing forces to discussing energy, another complex term. The relationship between the potential energy for covalent bond formation and the noncovalent attraction of two atoms as a function of distance is shown in general form in Figure 2.4.2 below.

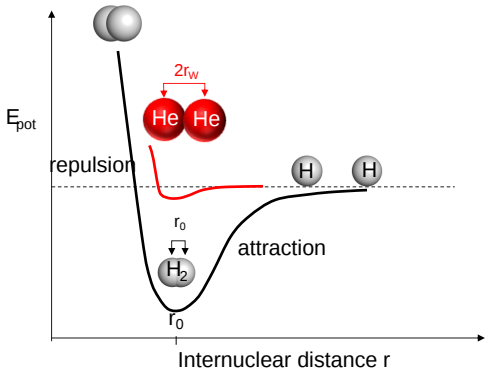


Figure 2.4.2: Potential Energy for Covalent and Noncovalent interactions

The curve in black shows the shape of E_{pot} vs. r for forming a covalent bond between H atoms. The Morse potential energy function is used to model energy as a function of r for simple diatomic molecules. The red line shows the shape of E_{pot} vs. r for the noncovalent attraction of two He atoms through induced dipole-induced dipole interactions. It is modeled using the Lennard-Jones (6-12) potential function (see below). Each has an optimal r_0 (the bond length for H_2 and two times the van der Waals radius, r_W, of each He in 2He). The energy required to break the induced dipole-induced dipole interactions between He atoms is very small, which accounts for the fact that liquid He, in which many He are interacting, only exists at very cold temperatures (boiling point = -269 Celsius).

Although the graph for H_2 shows the relationship between the potential energy and r_0 for the covalent bond, in reality, the sources of stability of any covalent bond are complex and require, in addition, a term for the kinetic energy of the electron. Fundamentally, the strength of a covalent bond is best described using quantum wave functions for the system. The average single covalent bond strength depends on the atoms bonded and varies between 30-120 kcal/mol (125-500 kJ/mol), a factor of 4.

Another confusing feature when discussing noncovalent interactions is that while discussing forces (like the electrostatic force), we often draw graphs of energy E vs r, the distance between two interacting particles. Let's briefly examine the relationship between potential energy (E_{pot}) and force for the electrostatic force given by Coulomb's Law by using a more familiar example, the next gravitational force of a stationary ball placed at various points on a hill, as illustrated in Figure 2.4.3.

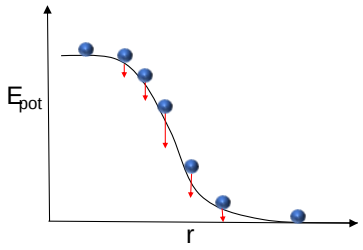


Figure 2.4.3: Potential Energy vs. r for a ball on a hill

Assume that the ball is motionless at each position in the diagram, and only potential energy is considered. The red arrows (vectors) represent the relative *net* downward force on the ball at each position. The *net* downward force at the top and bottom of the hill is zero. As the *slope* of the hill increases, the net downward force increases. The force is directly proportional to the slope (dE/dr), or simply:

$$F = -\frac{\Delta E}{\Delta r} = -\frac{dE}{dr} \tag{2.4.2}$$

Now let's apply this same relationship to Coulomb's Law for the force. Rearranging gives

$$dE = -\mathrm{F}\,\mathrm{dr} = -\frac{\mathrm{k}\mathrm{q}_1\mathrm{q}_2}{\mathrm{r}^2}\mathrm{dr} \tag{2.4.3}$$

Using calculus and integrating both sides of the equations gives this general relationship between E and r for the electrostatic forces:

$$E = \mathrm{k}\mathrm{q}_1\mathrm{q}_2\left(\frac{1}{\mathrm{r}}\right) \tag{2.4.4}$$

A graph of E_{pot} vs r for the electrostatic force is shown in Figure 2.4.4. Note that the curves are hyperbolic (1/r) functions of r. There are attractive OR repulsive components.

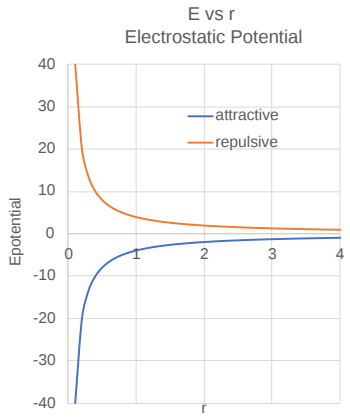


Figure 2.4.4: Electrostatic potential vs distance r

An equation for E_{pot} vs r for the induced dipole-induced dipole interactions can also be derived. For this interaction, E_{pot} has a different dependency on r and has both an attractive (E_{pot} α $-1/r^6$) AND repulsive term (E_{pot} α $+1/r^{12}$), which are added together. This potential is called the Lennard-Jones or 6-12 potential. Figure 2.4.5 shows the total and component attractive and repulsive terms E_{pot} vs r. Note how similar these curves are to the graphs for electrostatic energy.

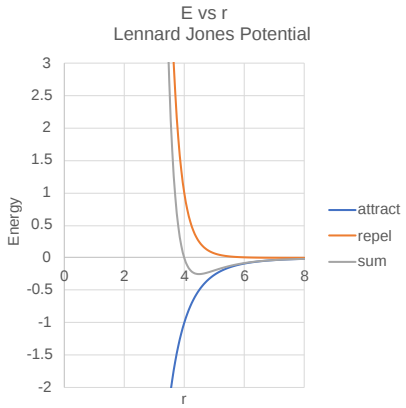


Figure 2.4.5: Lennard Jones Potential

Now, let's look at the noncovalent interactions more carefully using examples of small and big molecules.

2.4.2: Ion-Ion

All introductory chemistry and biology textbooks differentiate ionic and covalent bonding. Ionic bonding occurs between fully charged species. Some ions are monatomic (like Na^+ or Cl^-), formed from gaining or losing electrons. Others are polyatomic (like ammonium - NH_4^+ or acetate - CH_3COO^-, generally formed from molecules gaining or losing protons in Brønsted acid/base reactions. Polyatomic ions are also called molecular ions. An example of the monatomic salt NaCl and the molecular salt

ammonium acetate are shown in 2D Lewis structure and molecular modeling representations (spheres and sticks) in Figure 2.4.6: below.

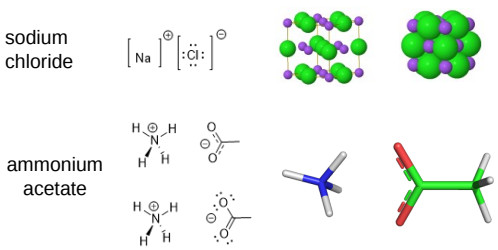


Figure 2.4.6: Representations of monatomic and polyatomic/molecular salts

Now, an intramolecular ion-ion interaction can form within a larger molecule if a negatively charged group in the molecule comes close enough in 3D space to a positively charged group in the same molecule. In contrast to the above examples, the ion-ion interactions within large molecules like proteins do not occur within a large lattice of ions held together by multitudes of similar ionic bonds. Rather, a single ion-ion interaction could persist in a larger molecule that is held together by many other noncovalent interactions. An ionic bond between a single monatomic or polyatomic cation and an anion would not exist in an aqueous solution as long as the species would dissociate into separate ions solvated by water. Hence, the ion-ion interaction between charged groups within a large molecule, like a protein, exists in a different environment than a solid crystal lattice. We often give it a different name, a ***salt bridge***, as the ion-ion interaction bridges distal parts of the larger molecule. We also categorize it as an **ion-ion** noncovalent attraction.

Figure 2.4.7 shows a salt bridge/ion-ion interaction (represented as a yellow line) between the side chains of two amino acids, aspartic acid (Asp) 67 ($-CH_2COO^-$, similar to acetate) and lysine (Lys) 69 ($-RCH_2NH_3^+$, similar to NH_4^+) in a protein, human lysozyme.

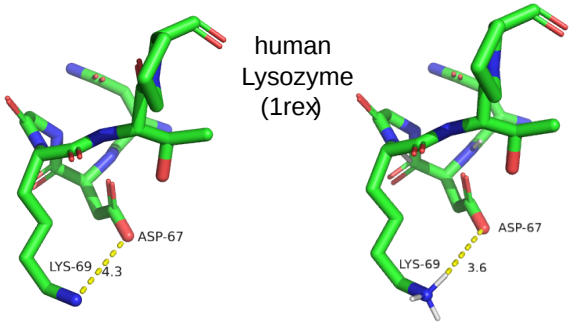


Figure 2.4.7: Salt bridge in human lysozyme

Figure 2.4.8 shows an interactive iCn3D model of a salt bridge/ion-ion interaction between the carboxylate side chain of Asp 67 and the amine side chain of Lys 69 in human lysozyme (1REX).

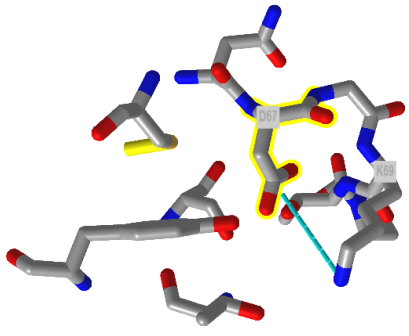


Figure 2.4.8: Salt bridge (represented as a yellow line) between Asp 67 and Lys 69 in human lysozyme (1REX). (Copyright; author via source).
Click the image for a popup or use this external link: https://structure.ncbi.nlm.nih.gov/i...1qpAtSs3CuvVs8

Most of the protein's atoms have been removed to simplify the structure. We haven't studied proteins yet, but to a first approximation, they are polymers consisting of amino acid monomers. The polymer's backbone contains a repeating amide group with an N-H hydrogen bond donor and a C=O hydrogen bond acceptor. Each amino acid contains an R group side chain oriented away from the backbone. The R groups can be fully charged, polar, or nonpolar.

This protein, containing 129 amino acids in a large polymer of over 1000 atoms, has just 10 salt bridges/ion-ion interactions within the most stable structure of the protein. The structure files that contain the x,y, and z coordinates of the atoms in a large biomacromolecule like a protein usually don't give coordinates for hydrogen atoms in the structure since they are too small to detect by techniques such as x-ray crystallography or cryoelectron microscopy, which are used to determine the structure of large biomacromolecules. Computer programs can be used to add them so they can be visualized in modeling programs. The left molecule in Figure 2.4.7 shows a stick model of a small protein part containing a single salt bridge/ion-ion interaction. The blue represents nitrogen with a +1 formal charge in the side chain of lysine.

Hydrogen atoms have been added to the right molecule to illustrate the distance between adjacent atoms. Quantum calculations of actual electron density in molecular ions such as H_3O^+ and NH_4^+ (and charged amines) show that the electron density in these cations is shifted to the electronegative O and N atoms with electron deficiencies over the bonded H atoms (in contrast to the simpler ideal of formal charge), even though we state that the N in a charged amine has a positive formal charge.

The attractions decrease as the distance r between interacting groups increases past the optimal interaction distance. When modeling most noncovalent interactions in large molecules, programs generally use cutoff values of 5-6 Angstroms, beyond which the interactions do not contribute to stabilization. Given a fixed distance for comparison, the ion-ion interaction is the strongest.

2.4.3: Hydrogen Bond (H-bond)

The name hydrogen bond is a bit ambiguous, which leads to students misunderstanding it. It is **not** a covalent bond between two atoms, X and H, such as C-H and O-H in methane and water, respectively. Rather, it is a noncovalent interaction between a slightly positive H on an electronegative atom X and a slightly negative electronegative atom Y on another molecule or part of a large molecule. X and Y are electronegative atoms such as F, O, or N with lone pairs. The H on X-H (for example, O-H or N-H) is slightly positive (δ^+) since the X-H bond is polar covalent, and electron density in the bond is drawn toward the electronegative atom (for example, O or N). Given its small size compared to all other atoms, a slightly positive H can get very close to a lone pair on another molecule's slightly negative (δ-) electronegative atom Y (for example, O or N). Since r, the distance between the δ^+ H and δ^- N or O on two separate molecules is small, Coulomb's Law informs us that the attractive force is significant. This interaction is highly directional and distance-dependent, which accounts for the large range in relative strength (3-15 kcal/mol, 12-63 kJ/mol) for hydrogen bonds within large molecules.

Hydrogen bonds occur between hydrogen bond donors and acceptors. This is determined by looking at the slightly negative electronegative atoms in the two interacting molecules. An alcohol (ROH) can be either a hydrogen bond donor or acceptor, while a ketone (R)C=OR) can only be an acceptor since it has no slightly positive H. In a hydrogen bond between an alcohol and a ketone, the O-H on the alcohol is the hydrogen bond **donor,** while an O=C on the ketone is the hydrogen bond **acceptor**. This is illustrated in Figure 2.4.9 below.

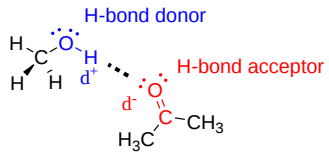


Figure 2.4.9: A hydrogen bond between a donor and acceptor

In a given hydrogen bond, the donor is the X-H with the slightly positive H.

Figure 2.4.10 shows multiple representations of a central water molecule hydrogen-bonded to four other water molecules. The left image shows lone pairs as purple spheres.

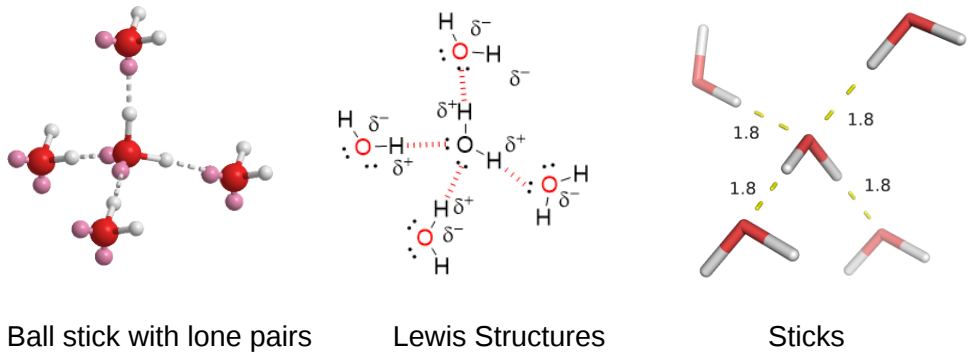


Figure 2.4.10: Multiple representations of hydrogen bonding among 5 water molecules

A common difficulty for students is identifying which hydrogen atoms in any structure can engage in hydrogen bonds. One way is to circle all δ^+ Hs in structures (i.e., those covalently attached to N or O) and see if there are any nearby δ^-: N or :O atoms close enough to form a hydrogen bond. Figure 2.4.11 shows a methanol molecule forming two hydrogen bonds with two different water molecules. Only 1 of the 4 Hs on methanol is δ^+ (circled in green). The others are bonded to the carbon through nonpolar covalent bonds.

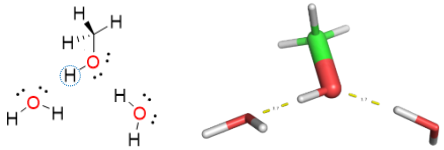


Figure 2.4.11: Hydrogen bond between methanol and waters

Hydrogen bonds are abundant in large molecules like proteins. They occur between backbone atoms, backbone and sidechain atoms, side-chain atoms, and protein atoms and water. Their strength depends on the magnitude of δ^+ and δ^- charges on the H bond donor and acceptor atoms, respectively, the distance r between them, and the bond angle. Three types of H bonds have been categorized based on their relative strengths, based in large part on the distance between the donor and acceptor:

- conventional (weakest of these three, so sometimes called weak), 2.4 to 12 kcal/mol (10-50 kJ/mol)
- strong or low barrier, 12 to 24 kcal/mol (50-100 kJ/mol), often called **short hydrogen bonds (SHB)**
- very strong or no barrier >24 kcal/mol (100 kJ/mol), (Frey et al).

In large proteins of known 3D structures, conventional H bonds are calculated by locating all donors and acceptors with 3 +/- 0.2 Angstrom (Å) from each other. Most structural files do not include H atoms, so the 3 Angstrom distance is measured from the centers of the electronegative atoms, typically N and O, involved in the hydrogen bond, as shown in Figure 2.4.12 (purple bracket).

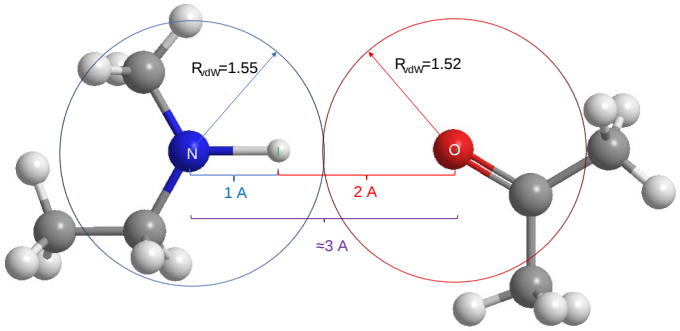


Figure 2.4.12: Hydrogen bonding distances between the center of a participating N with a H donor and an O with a H bond acceptor

Conventional H bonds vary between 2.8-3.2 A, which gives a distance range from the actual δ^+ hydrogen on the donor to the acceptor δ^- N or O (the red line below) of 1.8 to 2.2 A. Short H bonds are < 2.7 A, which is *smaller than the sums of the van der Waals radii of N and O* (blue and red circles above), suggesting that the bond has a covalent character (see below). Those between 2.5 and 2.7 A are strong, low-barrier, or short hydrogen bonds. Analysis of a large number of PDB structures of proteins shows many short hydrogen bonds characterized by these properties:

- the donor and acceptor electronegative atoms A and B are N or O
- r, the separation distance, is 2.3 A to 2.7 A
- the A–H–B angle is 135^0.

Detailed analyses of high-quality protein structures show one short hydrogen bond for every 16 conventional ones. They are found in proteins, protein-ligand complexes, and in DNA. They are involved in many aspects of molecular function.

It would seem likely that the δ^+ H atom, which is covalently attached to a heteroatom like O or N (A), and which is attracted to another heteroatom B, could be exchanged between the two heteroatoms as shown in the chemical equation below, where ---- represents an H bond.

A-H ----B ↔ A ----H-B

A very strong/no barrier H bond occurs if A and B are very close, have similar δ^- charges, and have similar pKa, so the H atom could be equally shared between A and B. It is represented by the representation below.

A ||| H ||| B

An example is FHF^- (F||| H |||F)$^-$ in which there is no barrier for the H to move from one heteroatom to another.

Thus, strong and very strong hydrogen bonds have some covalent bond character.. Quantum calculations show an overlap between the unoccupied antibonding σ*molecular orbital of X-H (the hydrogen donor) and the non-bonding lone electron pair molecular orbital of the hydrogen bond acceptor molecule.

Even though water is a simple and ubiquitous molecule, scientists still struggle to understand its properties. Lewis structures for water can explain only so much of its physical and chemical properties. However, look at Figure 2.4.13, which shows the electron density around water calculated using quantum theory.

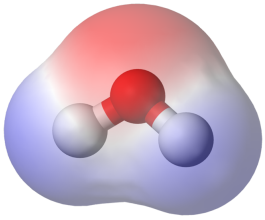


Figure 2.4.13: Electron density around a water molecule. https://commons.wikimedia.org/wiki/F...balls.png#file

Do you see any "rabbit ears" (i.e., lone pairs) emanating from the oxygen atom? Don't think so! Nevertheless, everyone still uses Lewis structures with lone pairs to explain the chemistry of water and other molecules. We present this figure before a discussion at the end of this section on the halogen bond, which requires understanding electron density around bonded atoms.

Now, let's look at some hydrogen bonds within a single protein molecule. Figure 2.4.14 hydrogen bonds (yellow dotted line) between serine (Ser) 24 (side chain $-CH_2OH$) and asparagine (Asn) 27 (side chain $-CH_2(C=O)NH_2$ of hen egg white lysozyme (1REX). As in the figures above showing salt bridges, two images are shown, one with polar H atoms added. Find the hydrogen bonds between side chains, side chains and backbone, and between backbone hydrogen bond donors and acceptors.

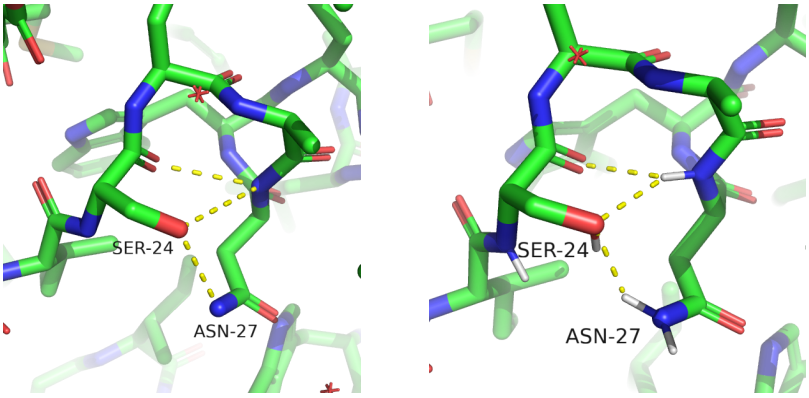


Figure 2.4.14: More hydrogen bonds in hen egg white lysozome (1REX)

Proteopedia has an excellent review of hydrogen bonds.

2.4.4: Dipole-Dipole

This interaction involves the alignment of permanent dipoles in molecules such that the geometric center of the δ^+ of one permanent dipole on one molecule is close to and aligned with the geometric center of δ^- of the permanent dipole on another. Figure 2.4.15 shows two acetone molecules interacting through dipole-dipole interactions. These molecules can't form hydrogen bonds with each other since they both contain just hydrogen bond acceptors.

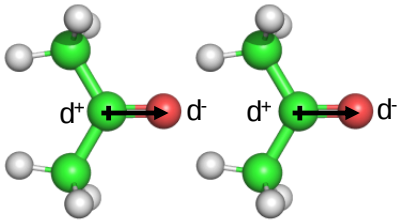


Figure 2.4.15: Attractions of two acetones through dipole-dipole interactions

The arrow represents the *molecule* dipole moment vector (as opposed to individual bond dipole moment for each polar covalent bond in the molecule). Note the difference in Figure 2.4.16. The molecular dipole is the vector sum of the bond dipoles.

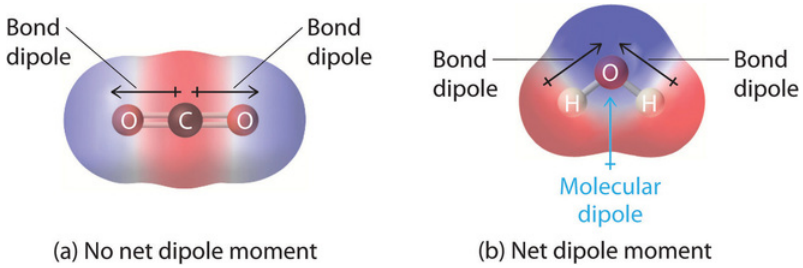


Figure 2.4.16: Difference between bond and molecular dipoles. https://chem.libretexts.org/Bookshel...bond%20dipoles.

None of the H atoms bonded to carbon in acetone are δ^+, so the molecules contain no H bond donors. Although they contain a δ^- oxygen, a hydrogen bond acceptor, two acetone molecules cannot hydrogen bond to themselves. However, they can form hydrogen bonds with water. Pure liquid acetone evaporates readily (BP 56^0 C) due to this lack of strong hydrogen bonds.

You can imagine two water molecules forming dipole-dipole interactions as well. However, tilting the molecule to align the lone pair on an O with the δ^+H on another water molecule and presto, you have a hydrogen bond. H bonds are often viewed as a special case of a dipole-dipole interaction.

Modeling programs can determine the charge on each atom of a large molecule like a protein and determine the geometric center and magnitude of overall + and - charge. A line between them represents the entire protein's permanent "dipole" moment. More simply, the molecular dipole is the vector sum of all of the individual bond dipole moments. Entire proteins have a net dipole

moment, which probably facilitates the interaction of the protein with other proteins or ligands. Figure 2.4.17 shows the net dipole moment for the protein carboxypeptidase A1 (2v77). This was calculated using the Protein Dipole Moments Server. Proteins, however, do have net charges (not considering any bound counterions), so the molecular dipole for a protein is a bit different conceptually from that of a small molecule. Nevertheless, it is a good way to quantify asymmetric charge distribution in large biomolecules. Asymmetric charge distributions would influence molecular properties.

Figure 2.4.17: Net dipole moment of the protein carboxypeptidase A1 (2v77)

2.4.5: Ion-Dipole

Figure 2.4.18 shows interactions between a Na^+ ion and the dipoles of multiple water molecules.

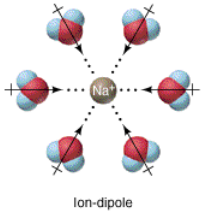


Figure 2.4.18: Ion-dipole interactions between Na^+ and water. https://chem.libretexts.org/Courses/...-Dipole_Forces

Figure 2.4.19 shows an interactive iCn3D model of the molecular ion sulfate SO_4^{2-} bound to a protein through its hydrogen bonding and ion-dipole noncovalent interactions with protein side chain and backbone groups in the sulfate binding protein from Salmonella typhimurium.

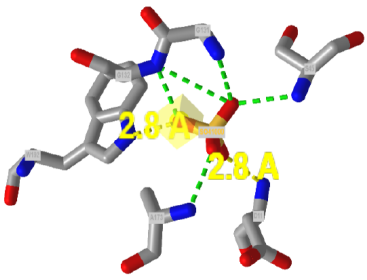


Figure 2.4.19: Sulfate bound to sulfate binding protein from S. typhimurium (1sbp). Hydrogen bonds are shown as green and yellow lines. (Copyright; author via source). Click the image for a popup or use this external link: https://structure.ncbi.nlm.nih.gov/i...JvqQuZZnvf3AU8

The SO_4^{2-} is buried within the protein. The green and yellow dotted lines show hydrogen bonds between the sulfate and amide N-Hs on the protein chain surrounding it and the protein's side chains. Modeling programs don't show lines depicting dipole-x interactions. The SO_4^{2-}, through its oxygen, can form hydrogen bonds with nearby donors.

Figure 2.4.20 shows an interactive iCn3D model of another example of a protein backbone and side chains ion-dipole interactions, this time with a Na^+ ion, a simple non-transition state metal ion, which can not form hydrogen bonds. The protein is tryptophan synthase from Salmonella typhimurium (6dz4). The red spheres represent water oxygen atoms (no hydrogen atoms shown).

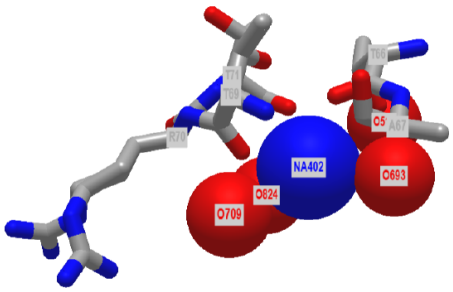


Figure 2.4.20: Sodium ion binding site in tryptophan synthase from Salmonella typhimurium (6dz4). Click the image for a popup or use this external link: https://structure.ncbi.nlm.nih.gov/i...pDnU36vXXPeyX7

The ions illustrated in these last two cases are not transition metal ions, whose interactions with ligands can best be considered using ligand field theory and the formation of covalent (dative) bonds between electron pair donors on nucleophilic side chain/main chain atoms and d orbitals on the transition metal.

2.4.6: Induced Dipole - Induced Dipole/Hydrophobic Interactions.

These noncovalent interactions occur when a temporary dipole, created by random fluctuations in electron density in one molecule, induces a temporary dipole in another molecule nearby. These interactions are weak and can easily be broken by raising the temperature. Induced dipole-induced dipole interactions allow nonpolar gases like He, N_2, O_2, and CH_4 to be liquefied, but only at higher pressures and/or low temperatures to force the molecules close enough and slow them down enough for sufficient interactions to occur to liquefy the molecules. Although individually weak, the larger the molecules, the greater the extent of induced dipole-induced dipole interactions and the stronger the interactions among molecules. This is reflected in the fact that methane, CH_4, is a gas at room temperature, octane, C_8H_{18} is a liquid, and $C_{30}H_{62}$ is a solid.

Figure 2.4.21 shows induced dipole interactions between two molecules.

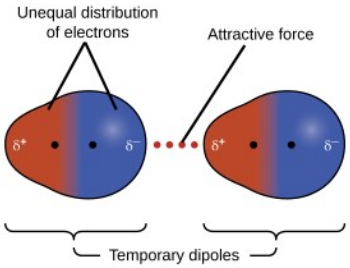


Figure 2.4.21: Induced dipole-induced dipole interactions. https://boisestate.pressbooks.pub/ch...ecular-forces/

Induced dipole-induced dipole interactions are important among large biomolecules as well. Most biologists and probably biochemists prefer to use the words hydrophobic interactions (but not hydrophobic forces) instead of the longer and more formal induced dipole-induced dipole interaction. We will also try to use the more commonly used term within the biochemistry community.

Figure 2.4.22 shows an interactive iCn3D model of a hydrophobic cluster around the side chain of a hydrophobic amino acid, valine 143, in human **carbonic anhydrase II (4ca2)**. Val 143 is highlighted in yellow and shown with normal atom (CPK) colors. White to green indicates nonpolar amino acids, while dark blue indicates polar ones.

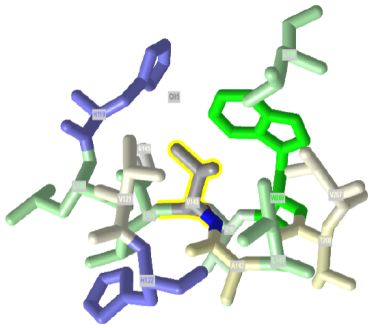


Figure 2.4.22: Hydrophobic cluster around the side chain of a hydrophobic amino acid, valine 143 in human carbonic anhydrase II (4ca2). Click the image for a popup or use this external link:https://structure.ncbi.nlm.nih.gov/i...6rgjajJsg3Xvg8

You can see that the side chain of Val 143 (highlighted in yellow) is surrounded by nonpolar amino acids. If the structure were rendered in spacefill instead of sticks, Val 143 would be closely packed to maximize induced dipole-induced dipole (hydrophobic)interactions.

Induced dipole-induced dipole interactions also occur between polar molecules, but they are weaker than the hydrogen bonding and dipole-dipole interactions between them.

2.4.7: Pi stacking

Aromatic rings stacked over each other can interact through induced-induced dipole (hydrophobic) and dipole-induced dipole interactions. These interactions can depend on the presence of heteroatoms in the aromatic ring. Figure 2.4.23 shows an example with benzene in which a staggered arrangement of the rings is more attractive.

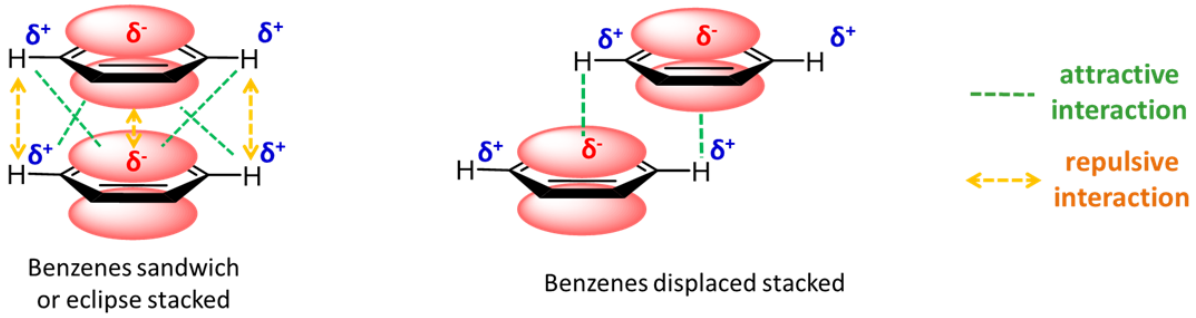


Figure 2.4.23: Pi stacking in benzene. https://chem.libretexts.org/Bookshel...g_interactions

For a biological example, everyone is familiar with the structure of B-DNA in which the bases A, G, C, and T point inward perpendicular to the double helix axis and are stacked over each other.

Figure 2.4.24 shows an interactive iCn3D model of a short stretch of DNA with a sugar-phosphate backbone and bases colored in magenta and cyan. Five bases on one strand are shown in stick and atomic color to show the pi-stacking interactions of the aromatic ring.

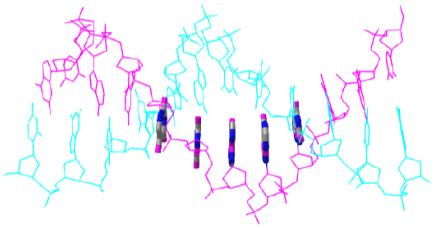


Figure 2.4.24: Pi stacking in B-DNA (5t4w). Click the image for a popup or use this external link: https://structure.ncbi.nlm.nih.gov/i...euHaVfXwk18JJ8

Pi stacking also occurs in proteins. Figure 2.4.25 shows an interactive iCn3D model of two sets of pi stacking interactions in the protein arginine kinase (1M15). The aromatic side chains involved in pi stacking are shown in cyan.

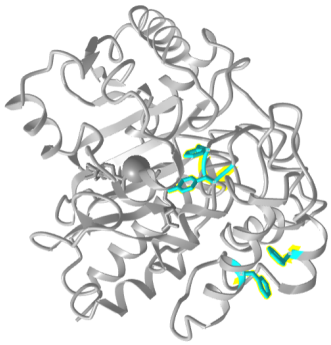


Figure 2.4.25: Pi stacking in Arginine Kinase (1M15). Click the image for a popup or use this external link: https://structure.ncbi.nlm.nih.gov/i...owSDJ2DUqtwk8A

2.4.8: Cation - Pi

Figure 2.4.26 shows an interactive iCn3D model of a specific example of an ion-induced dipole interaction (called a cation-pi interaction) between a sodium ion (blue sphere) and the aromatic ring of the side chain tryptophan (cyan) in hen egg white lysozyme (1lpi).

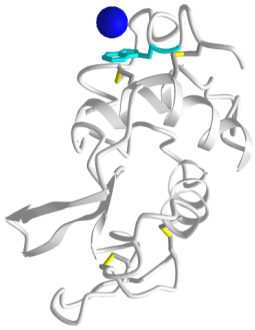


Figure 2.4.26: Cation-Pi stacking (ion-induced dipole) in hen egg white lysozyme (1lpi). Click the image for a popup or use this external link: https://structure.ncbi.nlm.nih.gov/i...13gw1hmVYrBL8A

Example 2.4.1

For another example of a cation-pi interaction, open up iCn3D with 1REX and view the interaction of lysine (K1) side chain with the nonpolar aromatic ring of phenylalanine (F3).

Solution

https://structure.ncbi.nlm.nih.gov/i...4d3wXXsSEYhgv7

Here are some more examples.

Exercise 2.4.1

Select the link below to answer the following questions.

1. What type of noncovalent interaction best describes the red dotted line in the structure?

2. What type of noncovalent interaction best describes the red dotted line in the structure?

Answer

1. cation-pi
2. pi stacking

2.4.9: Halogen Bond

Lastly, we come to the halogen bond. You might ask if there are halogens found in proteins. The answer is no (until one is found!), but halogenated molecules (drugs, xenobiotics, toxins) bind proteins. Consider the C-X bond where X is a halogen. The electronegativity of C is 2.56, while the halogens have these electronegativity values: F (3.98), Cl (3.16), Br (2.96), and I (2.66). Compare these to oxygen (3.44) and N (3.04). Covalent bonds between two bonded atoms whose electronegativity differences are between 0.4 and 1.8 are considered polar covalent, so C-F, C-Cl, and C-Br are considered polar covalent. The C-I bond is the longest and iodine is the most polarizable of these halogens. An alkyl halide with a C-I bond can undergo S_N2 nucleophilic substitution reactions, with I^- an excellent leaving group. Hence the C-I bond behaves somewhat as a polar covalent bond.

Nevertheless, quantum calculations show that the electron density is not uniformly spread around the X halogen in a C-X bond but rather is pulled more toward the C, leaving the distal *end* of the halogen depleted in electron density and slightly positive. This region of relatively depleted electron density is called the σ-hole. Color-coded renderings of the electron density of the halogen involved in a C-X bond show the halogen atom to have bands (like Jupiter) with the more negative electrostatic potential (represented in blue) closest to C and the more positive potential, the σ-hole (represented in red), at the end farthest from the C atom. Calculations show that this effect is greatest for the heavier halogens (Br, I) with longer C-X bonds. The halogen's slightly positive σ-hole can act analogously to a hydrogen bond donor in its interactions with nearby δ^- :O and :N atoms/lone pairs. This might take a while to grasp. You have always heard that, in general, the halogens are more electronegative than carbon and would hence always be δ^- when bonded to it. This case is similar to our chemical intuition about lone pair "rabbit ears" on oxygen, which quantum calculations show not to be an accurate representation of the electron density (see Fig 2.4.13).

Figure 2.4.27 shows the electrostatic potential on a halogen X atom covalently attached to a carbon in two different molecules, CF_3-I and :NC-Br. The red distal end is the σ-hole relatively depleted in electron density and with a higher, more positive electrostatic potential. (This is opposite the usual coloration that biochemists use in which oxygen (δ^- or fully -) is colored red and nitrogen (in a protonated amine with a positive charge) is shown in blue.)

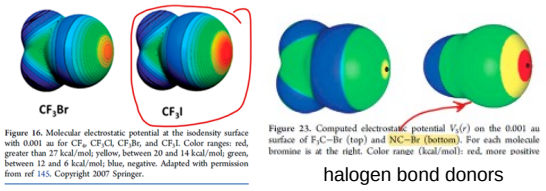


Figure 2.4.27: Electrostatic potential on a halogen X atom on CF_3-I and :NC-Br

Figure 2.4.28 shows a molecule with a carbonyl (a hydrogen bond acceptor with a δ^- :O) interacting with another molecule through either a hydrogen bond or a halogen bond. Again, the red distal end of the halogen X is the σ-hole relatively depleted in electron density.

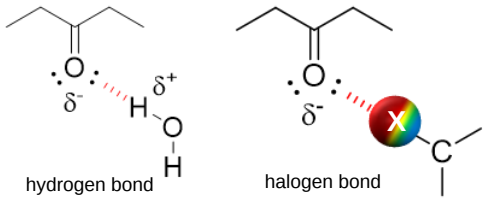


Figure 2.4.28: Comparison of a hydrogen and halogen bond (noncovalent interaction)

Medicinal chemists use halogen substituents on drug molecules to alter drug binding specificity, membrane diffusion, and $t_{1/2}$. Increasingly, they use halogen bonds in rational drug design to increase drug affinity to target proteins.

Figure 2.4.29 shows an interactive iCn3D model below shows the interaction of a haloaminopyrimidine inhibitor bound to its binding site on the c-Jun N-Terminal Kinase (JNK) protein (2P33).

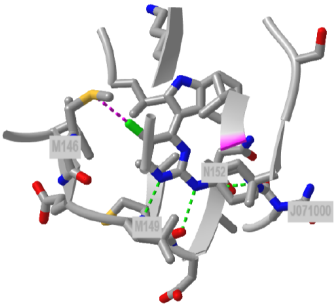


Figure 2.4.29: Haloaminopyrimidine inhibitor bound to its binding site on the c-Jun N-Terminal Kinase (JNK) protein (2P33). Click the image for a popup or use this external link: https://structure.ncbi.nlm.nih.gov/icn3d/share.html?H4Wabj8my3VsYLSi6

Note that the sulfur of methionine is forming a halogen bond with the Cl atom. Although the electronegativity of sulfur is 2.58, close to that of carbon (2.55), sulfur is larger and more polarizable, so it also develops a slightly positive σ-hole distal to the carbon atom. Analysis of PDB files shows that S--O interactions are common in proteins and most likely impact protein stability.

Ultimately, all ensembles of molecules/ions reach a low, if not the lowest, energy state under a given set of conditions. Noncovalent attractions are maximized, and repulsions are minimized to achieve this state. Consider, for example, solid sodium chloride held together by ionic bonds. The ions are closest packed (face-centered cubic). They cannot get closer together (packing density of about 74%) as simple packing considerations, repulsive electrostatic forces, and collective van der Waals interactions would prevent it. Each Na^+ is surrounded by 6 Cl^- ions and vice versa.

When large molecules like proteins assume a low energy state, they maximize the attractive noncovalent interactions described in this section while minimizing repulsive ones within a molecule (in given solvent conditions). Packing density reaches similar values to the closest packed spheres (NaCl, for example). Figure 2.4.30 shows a slice through a protein and the crystal lattice of NaCl. The gray circles on the protein show the faces of the sliced atoms. They are superimposed on the surface of the protein shown in colored spheres. If you took a series of cross-sectional slices throughout the protein, you would get a better picture of packing density than a single slice alone. Collective van der Waals interactions are found among all atoms and ions in a protein, which accounts for the closest packing of most atoms, polar and nonpolar, with the packed protein structure.

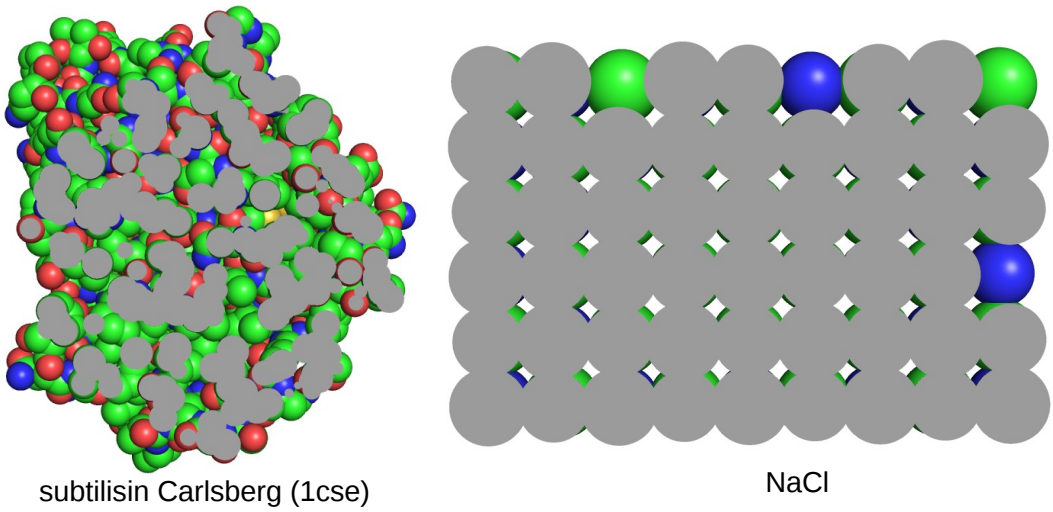


Figure 2.4.30: A slice through a protein and through the crystal lattice of NaCl

Here is a link to a JSmol tutorial by David Marcy et al, An Introduction to Chemical Bonds and Protein Structure

2.4.10: Summary of Noncovalent Interactions in Biomolecules

It is difficult to understand noncovalent interactions among small molecules, let alone within solvated and densely packed proteins. To help quantify strong noncovalent interactions involving amino acid *side chains*, Xie et al. studied amino acids in the gaseous phase using quantum mechanics. Here are some general conclusions (Xie et al. PLoS ONE 10(9): e0137113. https://doi.org/10.1371/journal.pone.0137113. Creative Commons Attribution License)

- Ion-Ion (salt bridge) interactions between acidic amino acids side chains (Glu^- and Asp^-) and alkaline amino acids side chains (Arg^+, Lys^+, and His^+) are the strongest residue-residue interactions. However, this type of interaction may be weakened by solvation effects and broken by lower pH conditions.
- Cation- interactions between protonated amino acid side chains (Arg^+, Lys^+, and His^+) and aromatic amino acid side chains (Phe, Tyr, Trp, and His) are 2.5 to 5-fold stronger than common hydrogen bond interactions and are less affected by the solvation environment.
- Amide bridge interactions, shown in Figure 2.4.31 below, which contain two hydrogen bonds between the two amide-containing amino acid side chains (in the amino acids Asn and Gln) are three times stronger than hydrogen bond interactions, which are less influenced by the pH of the solution.

Figure 2.4.31: An "amide" bridge (Xie et al., ibid)

- Ten of the twenty natural amino acids are involved in salt bridge, cation, or amide bridge interactions, often playing important roles in protein-protein, protein-peptide, protein-ligand, and protein-DNA interactions.

Secondary Interactions in Protein Structure and Function - New Findings

Recent Updates (01/7/24). Another computational study was done to categorize the noncovalent interactions between proteins and small molecules (drugs, inhibitors) that bind to them. These small molecules are generically called **ligands**, a term used in studying transition metal complexes. They studied 11,016 unique structures in the Protein Data Bank of small-molecule ligands bound to proteins. A histogram displaying the number of each type of interaction between small ligands and proteins is shown in Figure 2.4.32 below.

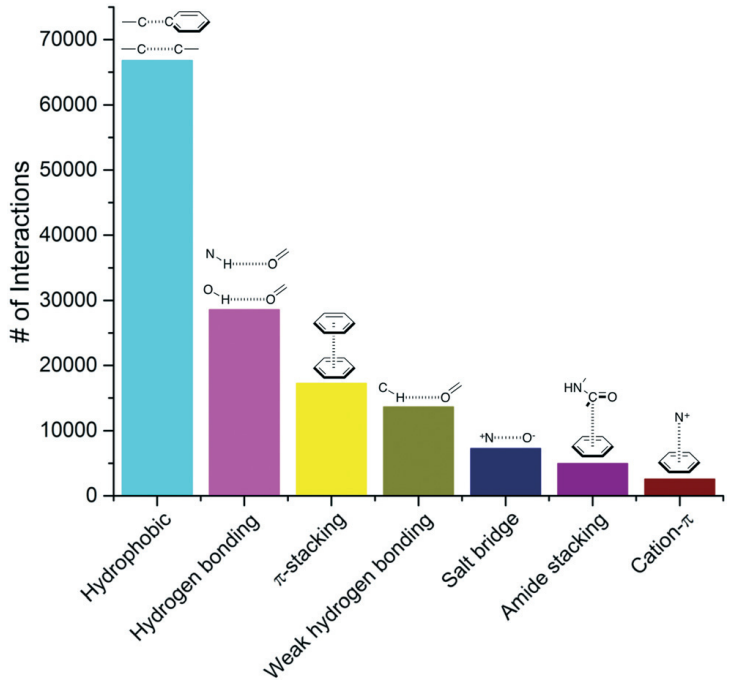


Figure 2.4.32 Frequency distribution of the most common non-covalent interactions observed in protein–ligands extracted from the PDB. de Freitas and Schapira. *Med. Chem. Commun.*, 2017, **8**, 1970-1981. **de Freitas and Schapira.** DOI: 10.1039/C7MD00381A (Research Article)

Two new interactions are shown: the **weak,** better called **nontraditional, hydrogen bond** and amide stacking. Amide stacking is readily understandable as an interaction between the amide's slight positive carbonyl carbon and the aromatic ring's electron-dense pi cloud. They are part of a group of interactions based on "**secondary forces**" between a ligand and protein and within proteins and carbohydrates, which we will explore in a future chapter. They include main chain interactions (nontraditional H bond, chalcogen bonds, and n→π*) and interactions with aromatic side chains. These secondary "forces" are shown in Figure 2.4.33 below.

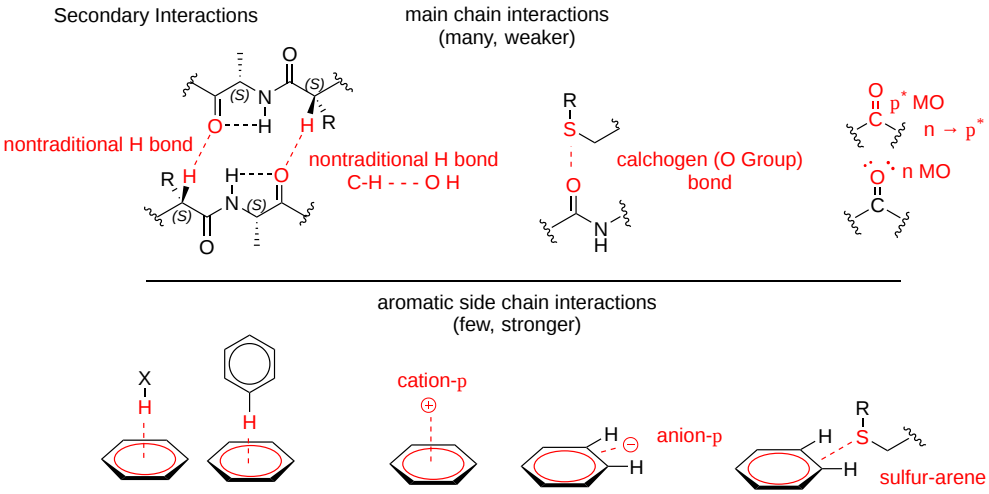


Figure 2.4.33 Secondary Interactions in protein (adapted from Newberry and Raines, *ACS Chem. Biol.* 2019, 14, 8, 1677–1686.

You probably find the nontraditional hydrogen bond more troubling. The hydrogen bond donor is a carbon atom attached to hydrogen, and a hydrogen bond acceptor is the carbonyl oxygen. We have stated that a C-H bond does **not** engage in a hydrogen bond. We assumed that a C-H bond is sufficiently nonpolar, so the carbon atom does not have a slight negative charge, which leaves hydrogen without a slight positive. Yet it appears that *there are many C–H···O weak hydrogen bonds between ligands and proteins and within proteins*.

C has an electronegativity of 2.5 and H 2.2 with a difference of 0.3, much smaller than between N and H (3.04-2.2 =0.8). If the electronic environment around the carbon enhances its slight negative charge, then you could imagine that a C-H could be a hydrogen bond donor. The median distance of the C–H···O nontraditional hydrogen bond was 3.4 Å, which is 0.4 Å longer than traditional hydrogen bonds (N–H···O, N–H···N, O–H···O), with an angle of around 130° (compared to an optimal of 109.5^0. The C_α–H···O=C interactions are about one-half the strength of an NH···O=C hydrogen bond. Hence, they are weak. In the rest of this book, we rarely see a nontraditional C-H bond as a candidate for a hydrogen bond. Nevertheless, it is important to mention it, given their prevalence.

The other main chain interaction, n → π^*, might look like a dipole-dipole interaction. It has its roots in the molecular (not atomic) orbitals for carbonyls in the backbone. Simple molecular orbital (MO) theory predicts the general energy levels for the molecular orbitals. The binding orbitals (σ for the sigma bond and π for the pi bond are filled with electron pairs and stabilize the molecule. The nonbonding electrons in the carbonyl O are in nonbonding orbitals n (neither stabilizing nor destabilizing). The antibonding σ* and π* orbitals on the adjacent carbonyl are unfilled and have higher energy. (Remember that if you bring two atomic orbitals (like 1s and 1s from 2 hydrogen atoms) to form an energy-stabilized covalent bond, you get 2 MOs (σ and σ*). The relative energy of these MOs for carbonyl is shown below in Figure 2.4.34:

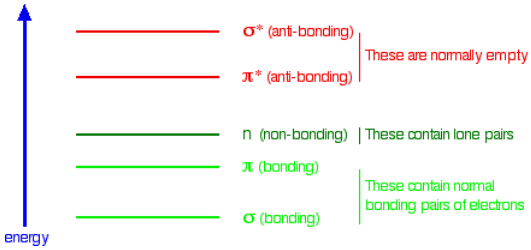


Figure 2.4.34: Relative energy levels of molecular orbitals (not to scale). https://chem.libretexts.org/Bookshel...c_Spectroscopy

Figure 33 shows some aromatic interactions that occur from the side and not the top of the plane of the ring. These are understandable if you consider the 3D electrostatic potential map of benzene, as shown in Figure 2.4.35 below.

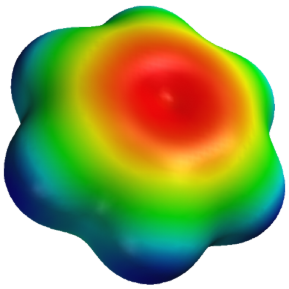


Figure 2.4.35: 3D electrostatic potential of benzene. https://en.wikipedia.org/wiki/File:B...-potential.png

Note that the sides are bluish, which represents more positive electrostatic potential, which would lead to the attraction of anion or S atoms with high electron density.

2.4.12: Summary

This chapter explores noncovalent interactions—often broadly labeled as intermolecular forces—that are central to understanding molecular behavior in biochemical systems. It begins by revisiting the concept of water as a solvent, emphasizing the "like dissolves like" principle and how the balance between solute-solvent, and solute–solute or solvent–solvent interactions, together with entropic contributions, determines solubility.

A detailed classification of noncovalent interactions follows, outlining key types such as ion–ion interactions (salt bridges), hydrogen bonds, dipole–dipole interactions, ion–dipole interactions, and induced dipole–induced dipole (hydrophobic) interactions. The chapter reinforces that all these interactions ultimately arise from the fundamental electrostatic forces described by Coulomb's law, with each type displaying distinct dependencies on distance and directionality.

The text then delves into the energetic landscape of these interactions by comparing potential energy versus distance profiles. Graphical models, including the Morse and Lennard-Jones potentials, illustrate how covalent bonds and noncovalent attractions achieve optimal stabilization at specific interatomic distances. This discussion is further enriched by analogies—such as a ball on a hill—to link changes in potential energy with the forces acting between particles.

Subsequent sections focus on specific examples within biomolecules. For instance, ion–ion interactions (or salt bridges) are examined through the lens of protein structure, where charged side chains contribute to stability. The complexity of hydrogen bonding is unraveled by differentiating between conventional, strong (low-barrier), and very strong hydrogen bonds, with attention given to geometric criteria and their role in molecular recognition and protein folding.

The chapter also highlights other interactions, such as dipole-dipole and ion-dipole forces, which are crucial for solvation and ligand binding, as well as aromatic interactions like pi stacking and cation–pi interactions, which underpin the stability of DNA and protein structures. A discussion on halogen bonds introduces the concept of the σ-hole, showcasing how anisotropic electron density distributions influence binding in drug design and molecular recognition.

Finally, the chapter touches on emerging secondary interactions—including nontraditional hydrogen bonds, amide stacking, and $n \rightarrow \pi^*$ interactions—that add further nuance to our understanding of protein structure and function. Together, these concepts illustrate how a network of noncovalent forces orchestrates biomacromolecules' precise assembly, stability, and dynamic behavior in aqueous environments.

In summary, this chapter integrates theoretical foundations, quantitative models, and real-world examples to provide a comprehensive framework for understanding how noncovalent interactions govern molecular behavior in biochemistry.

2.4.13: References

IUPAC. Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). Compiled by A. D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford (1997). Online version (2019-) created by S. J. Chalk. ISBN 0-9678550-9-8. https://doi.org/10.1351/goldbook.

https://chem.libretexts.org/Bookshel...g_interactions

https://boisestate.pressbooks.pub/ch...ecular-forces/

A low-barrier hydrogen bond in the catalytic triad of serine proteases, PA Frey et al, *Science* 264, 1927-1930 (1994)
DOI: 10.1126/science.7661899

2.5: Solubility in an aqueous world - The Hydrophobic Effect

Learning Goals (ChaptGPT o1, 1/25/25)

Below is a series of targeted learning goals designed to help junior and senior biochemistry majors develop a deep understanding of the thermodynamics of solute–solvent interactions and the hydrophobic effect:

- **Relate "Like Dissolves Like" to Biomolecular Solubility:**
  - Explain why nonpolar molecules (e.g., triacylglycerols, cholesterol esters) are poorly soluble in water and how liquid-liquid extraction exploits these differences.
- **Analyze Thermodynamic Contributions to Solubility:**
  - Differentiate between enthalpic and entropic factors that contribute to the free energy change (ΔG) for the dissolution or partitioning of biomolecules.
  - Interpret experimental data that show how increasing nonpolar chain length affects ΔG, and discuss the significance of per-methylene free energy changes.
- **Apply Free Energy and Chemical Potential Concepts:**
  - Use the relationships $\Delta G = \Delta H - T\Delta S$ and $\Delta G^\circ = -RT \ln K_{eq}$ to explain the spontaneity of solvation and partitioning reactions.
  - Define and relate chemical potential (μ) to free energy changes in a system at equilibrium.
- **Understand Partition Coefficients in Biphasic Systems:**
  - Describe how the partitioning of a solute between two immiscible phases (e.g., water and octanol) is quantified by the equilibrium partition coefficient (K_{part}) and how ΔG° can be calculated from experimental measurements.
- **Interpret the Hydrophobic Effect:**
  - Discuss the entropic penalty associated with forming an ordered water "cage" around a nonpolar molecule, and explain why this drives the aggregation of nonpolar molecules.
  - Analyze how releasing these structured water molecules upon phase separation contributes to a favorable free energy change.
- **Integrate Thermodynamic Data with Molecular Behavior:**
  - Evaluate experimental thermodynamic parameters ($\Delta\mu^\circ$, ΔH°, ΔS°) for the transfer of small amphiphiles (e.g., aliphatic alcohols) from their pure liquid to water and rationalize why enthalpic contributions may be favorable while entropy disfavors solubilization.
- **Connect Thermodynamics to Biological Function:**
  - Relate the principles governing the solubility and partitioning of biomolecules to their roles in biological membranes, drug diffusion across membranes, and overall cellular homeostasis.

These learning goals encourage an integrated understanding of both the quantitative thermodynamics and the qualitative molecular interactions that underlie the hydrophobic effect and solubility in aqueous environments.

2.5.1: Introduction

Many biomolecules, such as triacylglycerols, cholesterol esters, and waxes, are nonpolar. Other biomolecules, like proteins and many lipids, have polar and nonpolar parts. We know from experience that oil floats on the surface of water, showing that it is less dense than water and doesn't dissolve in water. You have also probably performed liquid-liquid extractions in chemistry labs, in which you utilized the solubility properties of nonpolar molecules to extract them from a mixture in water and transfer them to a more nonpolar phase, such as octanol or chloroform. To understand the stability of biomolecules that contain nonpolar parts in aqueous solutions, we need to understand not only the noncovalent interactions of the molecules with water (which we explored in Chapter 2.4) but also the thermodynamics of their molecular interactions in aqueous environments.

We have been taught and internalized the notion that "like-dissolves like." We anthropomorphize molecules to say nonpolar molecules "like" to be in nonpolar environments. We can rationalize solubility properties by examining a molecule's noncovalent attractive and repulsive interactions in an aqueous solution. Still, when we do so, we usually focus on enthalpic contributions to stability. What about entropy? We should consider net changes in noncovalent solute:solute, solute:solvent, and solvent:solvent interactions, as well as their thermodynamic contributions to overall stability. When we consider the thermodynamics of the solubility of molecules in water, we need to determine the ΔG, the free energy change, for all processes involved.

2.5.2: The Change in Free Energy (G) and Chemical Potential (μ)

ΔG, the free energy change for a reaction, determines the spontaneity and extent of a chemical or physical reaction. The free energy of a system depends on 3 variables, temperature T, pressure P, and n, the number of moles of each substance. For the latter, think of solute X on two different sides of a permeable membrane. If the concentration of X is the same on each side, as shown in Figure 2.5.1, the system is in equilibrium.

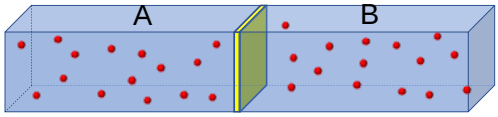


Figure 2.5.1: A system of a molecule in two compartments separated by a membrane

If the system is composed of two different parts, A and B, the system is at equilibrium (ΔG=0) if $T_A = T_B$, $P_A = P_B$, and the change in the absolute free energy per mole of A is $\Delta G_A/\Delta n = \Delta G_B/\Delta n$. More precisely, using simple calculus, we would discuss incremental changes in absolute free energy/mol, dG_A/dn for A, which is the chemical potential of A, μ_A) and dG_B/dn (μ_B) for B. At equilibrium, $dG_A/dn = dG_B/dn$. We will use the free energy G here but μ later in this section. G is the absolute free energy/mol (again chemical potential), where $G=G^o + RTln[A]$. The following equation can be written from the equations you used in introductory chemistry (which we reviewed in Chapter 1.3).

$$\begin{aligned} \Delta G &= \Delta G^0 + RTInQr \\ \Delta G &= \Delta H - T\Delta S \\ \Delta G^0 &= \Delta H^0 - T\Delta S^0 \\ \Delta G^0 &= -RTInKeq \end{aligned} \tag{2.5.1}$$

Now, let's apply this to the chemical equation for the solubility of a given solute in water. You eventually reach a saturation point if you add a sparing soluble hydrocarbon (HC) or sodium chloride to water. The salt solution is saturated with dissolved NaCl, and no further increase in NaCl (aq) occurs. The solution reaches saturation for a sparing soluble hydrocarbon, after which phase separation occurs.

Let's add a slightly soluble hydrocarbon liquid (HCL) drop into water, as pictured in the diagram below. At t=0, the system is not at equilibrium, and some of the HC will transfer from the pure liquid to water, so at time t=0, $\mathbf{\Delta G_{TOT}} < 0$. This is illustrated in Figure 2.5.2.

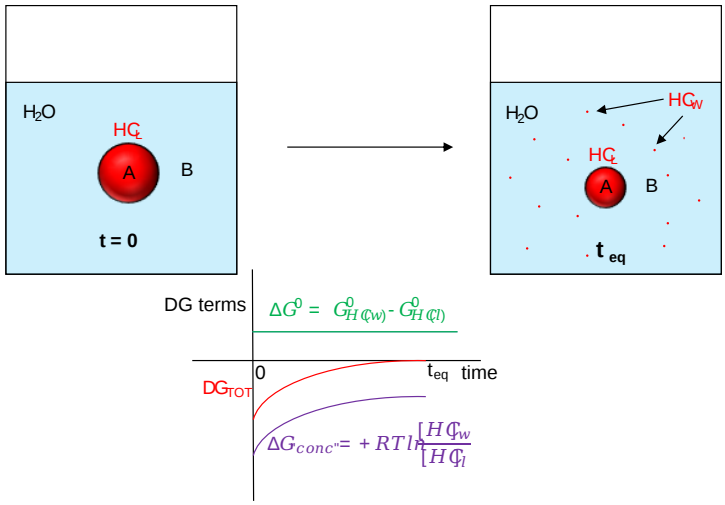


Figure 2.5.2: ΔG vs. time for interaction of a hydrocarbon with water

The following equations can be derived.

$$\begin{aligned}\Delta \mathrm{G_{TOT}} = (G_{\mathrm{HC-W}}) - (G_{\mathrm{HC-L}}) = \mathrm{G^0_{HC-W}} + RT\ln[\mathrm{HC}]_{\mathrm{W}} - \left(\mathrm{G^0_{HC-L}} + RT\ln[\mathrm{HC}]_{\mathrm{L}}\right) = \\ \Delta \mathrm{G_{TOT}} = \left(\mathrm{G^0_{HC-W}} - \mathrm{G^0_{HC-L}}\right) + RT\ln([\mathrm{HC}]_{\mathrm{W}} - \ln[\mathrm{HC}]_{\mathrm{L}}) = \\ \Delta \mathrm{G_{TOT}} = \Delta \mathrm{G^0} + RT\ln\frac{[\mathrm{HC}]_{\mathrm{W}}}{[\mathrm{HC}]_{\mathrm{L}}}\end{aligned} \tag{2.5.2}$$

Now, add a bit more complexity to the last example. Add a hydrocarbon x, to a biphasic system of water and octanol as shown in Figure 2.5.3. Shake it vigorously. At equilibrium, x would have "partitioned" between the two mostly immiscible phases.

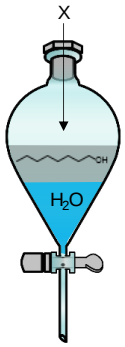


Figure 2.5.3: Use of separatory funnel for separating immiscible liquid phases

A simple reaction can be written for this system: X aq ↔ X oct.

If X is a hydrocarbon, $\mathbf{\Delta G} < 0$ for the reaction written above. Also, $\mathbf{\Delta G^o} < 0$, since this term is independent of concentration and depends only on the intrinsic stability of X in water compared to that of octanol. This simple equation holds:

$$\Delta \mathrm{G_{TOT}} = \left(\mathrm{G^0_{X-oct}} - \mathrm{G^0_{X-w}}\right) + RT\ln\frac{[\mathrm{X}]_{\mathrm{oct}}}{[\mathrm{X}]_{\mathrm{w}}} = \Delta \mathrm{G^0} + RT\ln\frac{[\mathrm{X}]_{\mathrm{oct}}}{[\mathrm{X}]_{\mathrm{w}}} \tag{2.5.3}$$

At equilibrium, ΔG=0 and the equation can be rewritten as:

$$\Delta \mathrm{G^0} = -RT\ln\frac{[\mathrm{X}]_{\mathrm{oct}}}{[\mathrm{X}]_{\mathrm{w}}} = -\mathrm{RTlnK_{part}} \tag{2.5.4}$$

where K_{part} is the equilibrium partition coefficient for X in octanol and water. This value can readily be determined in the lab. Just shake a separatory flask with a biphasic system of octanol and water after injecting a bit of X. Then separate the layers and determine the concentration of x in each phase. Plug these numbers into the last equation. You should be able to predict the sign and relative magnitude of $\mathbf{\Delta G^o}$ since it does not depend on concentration but only on the intrinsic stability of the molecules in the

different environments. K_{part} values are often determined for drugs since they often must diffuse across cell membranes to move into the cytoplasm, where they can act. Drugs, hence, must have a reasonable K_{part} to pass through the membrane but not so high that they are insoluble.

2.5.3: Introduction to the Hydrophobic Effect

Now let's ask this question: What are the enthalpic and entropic contributions to the **ΔG** for interacting a nonpolar molecule HC with water? For this section, we will replace ΔG with Δμ (the change in chemical potential, but we will use these terms interchangeably). Likewise, we will use this equation: $\Delta\mu^o = \Delta H^o - T\,\Delta S^o$.

Also, instead of framing the reaction as the dissolution of an organic molecule in water, we will frame it as the **transfer of a hydrocarbon X from an aqueous solution to the pure hydrocarbon liquid (HC) or**

$$X(aq) \leftrightarrow X(HC) \tag{2.5.5}$$

Figure 2.5.4 shows the standard free energies of transfer of a hydrocarbon X from an aqueous solution to a pure liquid hydrocarbon (HC), X (aq) ↔ X (HC). where

$$\Delta\mu^\circ = \mu^\circ x(HC) - \mu^\circ x(aq) \tag{2.5.6}$$

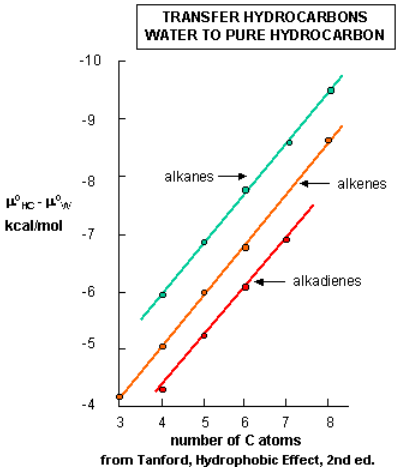


Figure 2.5.4: Standard free energies of transfer (μ^0) of a hydrocarbon X from aqueous solution to a pure liquid hydrocarbon (HC)

$\Delta\mu^o$ is less than 0 since transfer back to the pure HC is favored from a stability perspective. In each graph, $\Delta\mu^o$ is less than 0, and the value of $\Delta\mu^o$ decreases (gets more negative as you go up the y-axis, which shows increasingly negative values of $\Delta\mu^o$) in a linear fashion with increasing numbers of carbon atoms in the alkyl chain. Notice the lines are unbelievably straight and parallel. Nature is speaking to us in these figures. By determining the surface area of the hydrocarbon molecules and the decrease in $\Delta\mu^o$ with each added CH_2 (methylene group), one can calculate that the $\Delta\mu^o$ decreases by 25 cal/Å^2 (105 J/Å^2), per methylene added.

We expected that $\Delta\mu^o$ to transfer X to a pure liquid HC would be negative. We could get more information if we could determine both the entropic and enthalpic contributions. Such data is presented in the table below, which shows the transfer of short, single-chain alcohol X (an amphiphile with a polar head and a longer nonpolar "tail") from the pure liquid alcohol (ROH) to water (the opposite of the previous figures.)

$$X(ROH) \leftrightarrow X(W) \tag{2.5.7}$$

Thermodynamic Parameters for Transfer of Aliphatic Alcohol X from the **Pure Liquid to Water** at 25°C (enthalpy determined by calorimetry)

alcohol X	μ_w^0-μ_{ROH}^0 kcal/mol (kJ/mol)	H_w^0-H_{ROH}^0 kcal/mol (kJ/mol)	S_w^0-S_{ROH}^0 cal/deg mol (J/deg mol)	$(Cp)_w^0$-$(Cp)_{ROH}^0$ cal/deg mol (J/deg mol)
ethanol	0.760 (3.18)	-2.43 (-10.2)	-10.7 (-44.8)	39 (163)

n-propanol	1.58 (6.61)	-2.42 (-10.2)	-13.4 (-56.1)	56 (234)
n-butanol	2.4 (10)	-2.25 (-9.41)	-15.6 (-65.3)	72 (301)
n-pentanol (solubility 22g/L H_2O)	3.22 (13.5)	-1.87 (-7.82)	-17.1 (-71.5)	84 (352)

We expect the $\Delta\mu^o$ to be increasingly positive as the chain length gets longer and their solubilities in water become increasingly disfavored. What is perplexing about this data is not that the transfer of these ROHs to water is disfavored but that transfer is **enthalpically** favored (negative ΔH^0). This seems counterintuitive since it goes against the adage that "like dissolves like," as discussed earlier. From an enthalpic point of view, the amphiphiles prefer (albeit marginally) to be in water. What makes this reaction disfavored is entropy. The data shows that the nonpolar molecule would prefer not to be in the water because it is disfavored entropically.

At first glance, you might guess that the entropy should favor the movement of ROHs into the water since they could access a larger volume and have greater freedom of motion. Hence, there are more possible microstates for the ROH in water. However, this is only part of the process. What we haven't considered is the entropy of the water. A literal cavity must be created to accommodate a hydrocarbon in water. The creation of this more ordered cavity must be entropically disfavored (again because the process proceeds to a state with fewer microstates and lower positional entropy).

In the reverse process, transferring the hydrocarbon from water to the pure liquid dissipates the cavity, leading to more available microstates for the released solvent, bulk water. This entropic contribution favors the movement of a hydrocarbon from water to the pure hydrocarbon lipid. This "**hydrophobic effect**" is the main thermodynamic drive to move organic molecules out of water.

Image this scenario. When you place a hydrocarbon group into water, water seeks (admittedly an anthropomorphic term) to maintain its hydrogen bonding. Hence, it is forced into a more ordered structure around the HC to maintain its H-bonding, characterized by fewer microstates. We will explore the hydrophobic effect in greater detail in a future chapter.

How can we explain the favorable enthalpic contribution of placing a nonpolar molecule into water? Again, this goes against our adage of "like dissolves like". The negative ΔH suggests interactions among all the participants are more favorable when the nonpolar group is in water. One source of such interactions could be the highly structured water in the "cage" surrounding the nonpolar molecule. If it were more structured than bulk water, hence more "ice-like" in nature, then the formation of these extra H-bonds would contribute to the negative enthalpy change. When the nonpolar molecule is removed from the water, which proceeds with a positive ΔH, the "ice-like" water cage would "melt", which, like ice melting, is not favored enthalpically, as heat must be added. Heat energy must be supplied to break the H-bonds as ice changes state to liquid water. This molecular model to understand the thermodynamic data might be simplistic, but for now, let's use it.

2.5.4: Summary

This chapter examines the thermodynamics underlying the solubility of biomolecules in aqueous environments, focusing on understanding how nonpolar and amphiphilic molecules behave in water. It begins by revisiting the principle of "like dissolves like," illustrated by everyday observations such as oil floating on water and the practice of liquid-liquid extraction. These examples highlight that nonpolar substances (e.g., triacylglycerols, cholesterol esters, waxes) do not readily mix with water, while many biomolecules possess polar and nonpolar regions.

The discussion then shifts to a detailed exploration of the thermodynamic factors that govern solubility. Key concepts such as free energy (ΔG), chemical potential (μ), and their dependence on temperature, pressure, and concentration are introduced. The chapter emphasizes that favorable enthalpic interactions and entropic contributions determine the spontaneity of solvation processes. Using the example of transferring a hydrocarbon from water to a pure hydrocarbon liquid, the text derives equations that relate the free energy change (ΔG) to the equilibrium partition coefficient (K_part) through the relation $\Delta G° = –RT\ lnK_{part}$.

A significant portion of the chapter is dedicated to the hydrophobic effect. It explains that although the transfer of nonpolar molecules into water might be enthalpically favorable—owing to the formation of structured, “ice-like” water cages around the nonpolar moieties—the overall process is disfavored due to a substantial entropic penalty. This entropy loss arises from ordering water molecules that must occur to accommodate the nonpolar solute. Conversely, when nonpolar molecules aggregate or partition into a nonpolar phase, water is released from this ordered state, resulting in an overall favorable free energy change that drives many biological processes, such as membrane formation and protein folding.

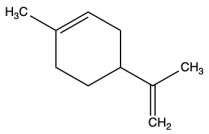
By integrating theoretical thermodynamics with experimental data, this chapter provides a comprehensive framework for understanding how enthalpic and entropic forces interplay to determine the solubility and partitioning behavior of biomolecules, a foundational concept in biochemistry.

2.6: Chapter 2 Questions

Section 1 Questions:

Q1) Based on the interactive figure 2.1.1 in the default view, do you hypothesize that water could enter the core of the micelle? Now, let's test your hypothesis! Open the interactive figure, and click Style → Surface Type → Solvent Accessibility. Based on the space-filled solvent accessibility map, do you see any openings for water molecules to enter through? Explain your answer.
A1) Hypothesis can range from no water is accessible, or water could freely occupy the "empty" space in the default view of the micelle. However, once the solvent accessibility filter is applied, it is clear that the micelle will exclude water from entering the hydrophobic core. Some areas of the micelle (the polar head groups) are able to for interactions with the water and therefore show up as red/yellow in this view, but it is key to note there are no pores or solvent-accessible spaces on the surface of the micelle.

Q2) The structure for Limonene, the compound that gives citrus fruits their classic smell is shown below. Examine the structure and answer the following questions.



a) Will Limonene form any associations with water molecules? Explain.
b) For our brain to register the citrus aroma, Limonene needs to bind to a surface protein receptor, what type of interactions could be taking place? Explain.
c) When Limonene binds to its receptor are the interactions from b) stabilized by enthalpy ΔH or entropy ΔS?

A2)
a) No, there are no dipoles or polar functional groups on limonene.
b) The methyl and ethyl functional groups would likely form hydrophobic bonds with nonpolar amino acids in the protein. Because there are no polar/dipole residues, Limonene cannot make hydrogen or ionic bonds.
c) Binding of Limonene to its surface receptor will likely be stabilized by entropy. When the nonpolar Limonene binds to a nonpolar region of the surface receptor, order water will be released, thus creating a +ΔS.

Q3) Consider the following reaction of a ***polar*** substance and water at room temperature (22°C):
$Glucose_{(s)} + H_2O_{(l)} \rightarrow Glucose_{(aq)}$
a) Estimate the enthalpy ΔH and entropy ΔS (+,-,≈0) for the reactants and product. Consider the order/disorder of the over all reaction as well as the net charge of the bond enthalpy.
b) Based on the Gibbs free energy equation ΔG = ΔH - TΔS, will this reaction be spontaneous, non-spontaneous, or at equilibrium?

A3)
a) $Glucose_{(s)} + H_2O_{(aq)} \rightarrow Glucose_{(aq)}$
ΔH: H-bonds H-bonds H-bonds ΔH ≈ 0
ΔS: Solid Liquid Solution ΔS = +

b) ΔG = ΔH - TΔS
= 0 - (+)
= -
This dissolution of polar substances into water is spontaneous.

Q4) Consider the following reaction of a ***nonpolar*** substance and water at room temperature (22°C):
$Methane\ (CH_4)_{(g)} + H_2O_{(l)} \rightarrow Methane\ (CH_4)_{(aq)}$
a) Estimate the enthalpy ΔH and entropy ΔS (+,-,≈0) for the reactants and product. Consider the order/disorder of the overall reaction as well as the net charge of the bond enthalpy.
b) Based on the Gibbs free energy equation ΔG = ΔH - TΔS, will this reaction be spontaneous, non-spontaneous, or at equilibrium?

A4)
a) $Methane\ (CH_4)_{(g)} + H_2O_{(l)} \rightarrow Methane\ (CH_4)_{(aq)}$

ΔH: None H-bonds H-bonds ΔH ≈ 0 or slightly -
ΔS: Gas Liquid Solution ΔS = -

b) ΔG = ΔH - TΔS
= 0/- - (-)
= +
This dissolution of nonpolar substances into water is non-spontaneous. This is primarily due to water forming an ordered cage around the nonpolar methane gas. In terms of enthalpy, the water cage creates a more favorable environment for methane than the pure gas in liquid water, thus the enthalpy can be considered slightly negative. However, the cage structure the water molecules form around the methane gas makes the system ordered. Therefore the overall ΔG for nonpolar substances solubilized with water is positive and therefore non-spontaneous.

Need to add in some reactions? (last chunk of section 1)

Section 2 Questions

Q1) Tyrosine is commonly found in the active sites of enzymes, as the unique structure of its R-group can act as either an acid or base.

a) The enzyme DcpS, an mRNA capping enzyme, utilizes a tyrosine residue as an active site acid, and measurements show that the tyrosine is 75% ionized. What must the local pH need to be for this to occur?
b) Another way for tyrosine to be used as an acid is to lower its pKa by creating weak interactions with the oxygen of its R-group using neighboring amino acids. What type of interaction(s) could achieve this goal?
c) One enzyme utilizing the strategy in b) is Glutathione-S-Transferase. Measurements show that the active site pH is 7.5, and the tyrosine residue is 95% ionized. What must the pKa of tyrosine be for this to occur?

A1)
a)

$$pH = pK_a + log\frac{[A^-]}{[HA]}$$

$$pH = 10.1 + log\frac{[75]}{[25]}$$

pH = 10.1 + (0.477)
pH = 10.58

b) Hydrogen bonding can create a change in the net dipole of the oxygen in tyrosine by making it more acidic and decreasing the pKa.
c)

$$pH = pK_a + log\frac{[A^-]}{[HA]}$$

$$7.5 = pK_a + log\frac{[95]}{[5]}$$

7.5 = pK_a + 1.28
6.22 = pK_a

Section 3 Questions

Q1) Your lab wants to study an enzyme that catalyzes a reaction inside the chloroplast stroma, which has a pH of 8.0 due to the proton gradient that pumps H^+ from the stroma to the thylakoid lumen.
a) Which of the buffers below would be the best choice to study this enzyme, in vitro? Explain your choice. Are there any other buffers that could work?
-Tricine: pKa 8.05
-TAPS: pKa 8.40
-MES: pKa 6.1
-Citrate: pKa 6.40
-HEPES: pKa 7.48
b) How many moles of the conjugate base form of HEPES would there be in 2.5 L of a 175 mM solution at pH 8.0?

A1)
a) Tricine would be the best choice as buffers work best at a pH closest to their pKa. TAPS or HEPES could work in a pinch if Tricine was not available, but remember, pH and pKa are log scales! So, while the pKa values might not seem that far from the intended pH of 8.0, on the log scale that is quite a difference in H^+ concentration.
b)

$$2.5L \times \frac{175mmoles}{L} \times \frac{1mole}{1000mmoles} = 0.4375mmoles$$

Now we want to determine what fraction of those moles are in the conjugate base form:

$$[A^-] + [HA] = 0.4375$$

$$[A^-] = 0.4375 - [HA]$$

$$pH = pK_a + log\frac{[A^-]}{[HA]}$$

$$8.0 = 7.48 + log\frac{[A^-]}{[HA]}$$

$$0.52 = log\frac{[A^-]}{[HA]}$$

$$3.31 = \frac{0.4375 - [HA]}{[HA]}$$

At pH 8.0, [HA] = 0.101 mmoles, which is 23% of the total moles of HEPES in the solution.

Q2) As discussed in Section 2.3, when CO_2 is inhaled, it reacts with water to form the weak acid carbonic acid, acidifying the blood. The reaction is given below for reference.
$CO_2 + H_2O \rightarrow H^+ = HCO_3^-$
Currently, the air you breathe contains about 0.04% CO_2. This number has risen from 0.03% in the 1960s and is projected to increase to 0.08% by 2100 if fossil fuel consumption remains at its current rate. (P_{CO2} =0.0003 atm, Keeling, 1960, P_{CO2} =0.0008 atm MIT System Dynamics Group, 2015).

Using the Ideal Gas Laws, there were 0.44 μM CO_2 in the 1960s, 0.60 μM CO_2 today, and as much as 1.2 μM CO_2 in 2100. The pKa of carbonic acid is 6.35 and the pH of your blood is 7.60.

a) What is the change in blood pH due to the increase in atmospheric CO_2 from the 1960s to today?
b) If you were to do the calculations for what the pH of the blood would rise to in 2100, you'd find it to be pH 7.3. Do you think the body would be able to compensate for this? Use the pKa of histidine to explain your answer.

A2)
a)

$$pH = pK_a + log\frac{[A^-]}{[HA]}$$

$$7.60 = 6.35 + log\frac{[A^-]}{0.6}$$

$$1.25 = log\frac{[A^-]}{0.6}$$

$$17.8 = \frac{[A^-]}{0.6}$$

$[A^-]$ = 10.7 μM

$$pH_1960 = 6.35 + log\frac{10.7}{0.44}$$

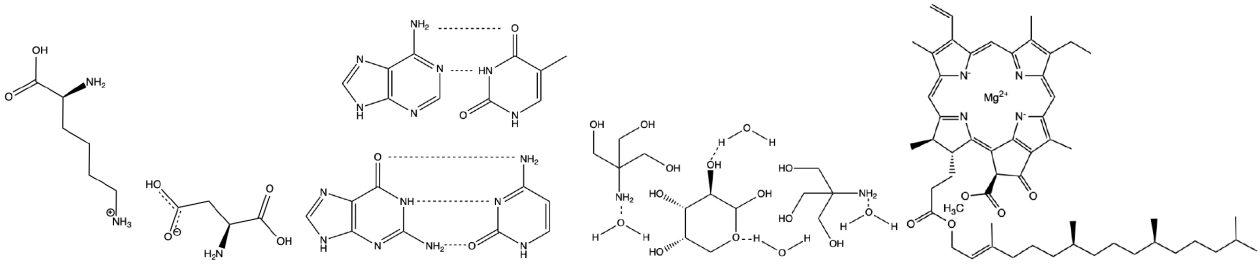
pH_{1960} = 6.35 + 1.39
pH_{1960} = 7.74

Now knowing the pH of the blood from 1960, the change is 7.74 - 7.6 = 0.14 pH units.

b) Yes the body would compensate by increasing the amount of carbonic acid in the blood to buffer the increase in free H^+. If the body did not do this, the protonation state of histidine would change and affect every histidine-containing protein in the blood.

Section 4 Questions

Q1) Categorize the following bonds as ion-ion, ion-dipole, dipole-dipole, H-bond, or hydrophobic, (some maybe be used more than once, or not at all).



A1)

Q2) You discover a new enzyme, "biochemase" and decide to crystalize the protein to determine if you can determine the role from its structure. You hypothesize the protein forms a homodimer when two identical subunits of a protein come together to form one functional structure. You identify two regions that you believe could be the dimer interface. The first region looks to contain several polar amino acids, whose R-groups are less than 5 Å apart, while the second section contains a large cluster of hydrophobic amino acids.

a) You perform some biochemical experiments and discover that when the **polar region** is removed, the enzyme is not able to self-assemble, but maintains two **intact separate structures**. What can you conclude about this region and the type of interactions that are stabilizing the dimer interface?

b) When you remove the **nonpolar** region, you notice the protein aggregates or crashes out of the solution due to an inability to fold correctly. What role can you hypothesize the nonpolar amino acids have in keeping the protein properly folded?

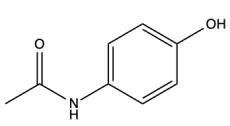
A2)

a) When the polar region is removed, the protein is still able to fold into two stable subunits, but unable to form its homodimer. Therefore we can conclude that the polar region is necessary for the dimer interface, but the protein can still form subunits without it present.

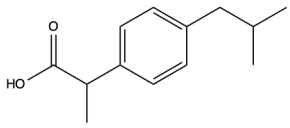
b Now, when the nonpolar region is removed the protein cannot form subunits or dimers. So, we can conclude that the nonpolar region is necessary for protein stability, and without this region present, the protein cannot fold properly. Most likely, the hydrophobic region forces the protein to fold in the correct way by forcing the nonpolar amino acids to the core, and the polar to the surface. This would then allow for a proper dimer interface to form.

Section 5 Questions

Q1) Acetaminophen (Tylenol) and Ibuprofen (Aspirin) are both common pain-relieving/fever-reducing drugs. However, their chemical properties differ, making acetaminophen more suitable for relieving headaches and fever, while ibuprofen can more effectively reduce pain. The pK_{part} for acetaminophen is 0.91, while the pK_{part} is 3.97. (DrugBank) The chemical structures for each are given below:



Acetaminophen



Ibuprofen

a) Using equation 2.5.4, determine the ΔG° for each compound, and hypothesize which is more soluble based on your answer and explain. Assume a normal body temperature of T = 37°C

b) With your answer from a) and the information reviews in chapter 1 on functional groups, and types of non-polar interactions in chapter 2, identity the regions of both acetaminophen and ibuprofen that can facilitate interactions.

c) Using your ΔG° calculations and functional group analysis, predict which compound is more likely to stay in the blood and bound to red blood cells, and which can rapidly diffuse across the cell membrane.

Q2) You are tasked with creating a solution of nonpolar and polar solvents to use for thin layer chromatography (a technique we will cover later), to separate lipids. Consider the following enthalpic and entropic values for these nonpolar and polar solvents. Using the information discussed in section 5, which of the following combinations of solvents will yield a homo

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CHAPTER OVERVIEW

3: Amino Acids, Peptides, and Proteins

Return to Fundamentals of Biochemistry Search Fundamentals of Biochemistry

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3.1: Amino Acids and Peptides

Learning Goals (ChatGPT o1, 1/25/25)

- **Understand the Role and Diversity of Proteins:**
  - Describe the various functions of proteins (structural, regulatory, enzymatic, transport, etc.) and explain why proteins are considered the most functionally diverse macromolecules.
  - Recognize that proteins are linear polymers of alpha-amino acids linked by peptide bonds.
- **Comprehend Alpha Amino Acid Structure:**
  - Identify the common structural features of an alpha amino acid (α-amino group, α-carboxylic acid, hydrogen, and a variable R-group).
  - Memorize the 20 naturally occurring amino acids along with their three-letter and one-letter abbreviations.
  - Explain how variations in the R-group confer different chemical properties (nonpolar, polar uncharged, acidic, and basic) to amino acids.
- **Master Peptide Bond Formation and Protein Primary Structure:**
  - Describe the mechanism of peptide bond formation via nucleophilic attack and the release of water.
  - Illustrate the concept of primary protein structure as the linear sequence of amino acids and appreciate the enormous diversity of possible sequences.
- **Analyze Amino Acid Classification and Side Chain Characteristics:**
  - Categorize amino acids based on their side chain properties (e.g., aliphatic vs. aromatic nonpolar, polar uncharged, acidic, and basic).
  - Discuss how side chain properties influence protein folding, stability, and function.
  - Interpret hydrophobicity (or hydropathy) scales (e.g., Kyte-Doolittle, Hopp-Woods) and relate these to protein topology (buried versus surface residues).
- **Grasp Stereochemistry and Chirality in Amino Acids:**
  - Explain the concept of chirality and why all naturally occurring proteinogenic amino acids (except glycine) are L isomers.
  - Differentiate between the D/L nomenclature and the R/S system, and understand why the D/L system is preferred in biochemistry.
- **Apply Acid-Base Chemistry to Proteins:**
  - Utilize the Henderson–Hasselbalch equation to predict the ionization state of amino acid side chains and terminal groups.
  - Interpret titration curves for amino acids and proteins and calculate the isoelectric point (pI) of proteins based on the ionizable groups present.
- **Examine Chemical Reactivity of Amino Acid Side Chains:**
  - Identify which side chains serve as hydrogen bond donors and acceptors, and explain their roles in catalysis and substrate binding.
  - Compare the nucleophilicity and basicity of key amino acid side chains (e.g., lysine, cysteine, histidine) and discuss factors (like electronegativity and steric effects) that influence reactivity.
  - Describe specific chemical reactions involving amino acid side chains, including acylation, Schiff base formation, and nucleophilic substitution reactions used in protein modification.
- **Explore Post-Translational Modifications (PTMs) and Their Biological Significance:**
  - List common PTMs (e.g., phosphorylation, acetylation, glycosylation, oxidation) and explain how these modifications alter protein structure and function.
  - Understand how aberrant or deleterious PTMs (e.g., glycation, carbonylation) can lead to altered protein activity and contribute to disease processes.

These learning goals are intended to help students integrate structural, chemical, and functional perspectives on proteins, preparing them for more advanced topics in enzymology, protein engineering, and molecular biology.

3.1.1: Introduction

Proteins are one of the most abundant organic molecules in living systems and have the most diverse range of functions of all macromolecules. Proteins may be structural, regulatory, contractile, or protective. They may serve in transport, storage, or membranes. They may be toxins or enzymes. Each cell in a living system may contain thousands of proteins, each with a unique function. Their structures, like their functions, vary greatly. They are all, however, polymers of alpha amino acids arranged in a linear sequence and connected by covalent bonds.

3.1.2: Alpha Amino Acid Structure

The major building blocks of proteins are called ***alpha (α) amino acids***. As their name implies, they contain a carboxylic acid and an amine functional group. The alpha designation indicates that these two functional groups are separated by one carbon group. In addition to the amine and carboxylic acid, the alpha carbon is also attached to hydrogen and one additional group that can vary in size and length. In the diagram below, this group is designated as an R-group. Within living organisms, 20 common amino acids are used as protein building blocks. They differ from one another only in the R-group position. The fully protonated structure of an amino acid (at low pH) is shown in Figure 3.1.1.

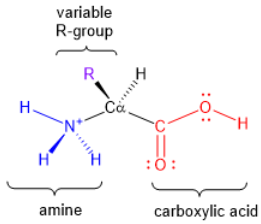


Figure 3.1.1: Generic Structure of an Amino Acid

The twenty common naturally occurring amino acids contain alpha-carbon, an amino, carboxylic acid, and an R group (or side chain). The R group side chains may be either nonpolar, polar uncharged, or charged, depending on the functional group, the pH, and the pKa of any ionizable group in the side chain.

Two other amino acids occasionally appear in proteins. One is selenocysteine, found in Archaea, eubacteria, and animals. Another is pyrrolysine, found in Archaea. Bacteria have been modified to incorporate two new amino acids, O-methyl-tyrosine and p-aminophenylalanine. The yeast strain *Saccharomyces cerevisiae* has been engineered to incorporate five new unnatural amino acids (using the TAG nonsense codon and new, modified tRNA and tRNA synthetases) with keto groups that allow chemical modifications to the protein. We will concentrate only on the 20 abundant, naturally occurring amino acids.

Proteins are polymers of monomeric amino acids with an amide link (also called a peptide bond) between the α-carboxylic group of one amino acid with the α-amine of the next one. Figure 3.1.2 shows the twenty naturally occurring α-amino acids as they would appear internally within a protein sequence. The squiggles show the connecting amide/peptide bond between adjacent amino acids. Students often assume that the α-amino and α-carboxylic acid groups within a protein sequence are free and not part of the peptide bond. This figure should help in resolving that misconception. The three-letter and one-letter abbreviations of each amino acid are shown, as well as the typical pKa values of side chains (R groups). It is important to memorize the three-letter and one-letter codes for the amino acids.

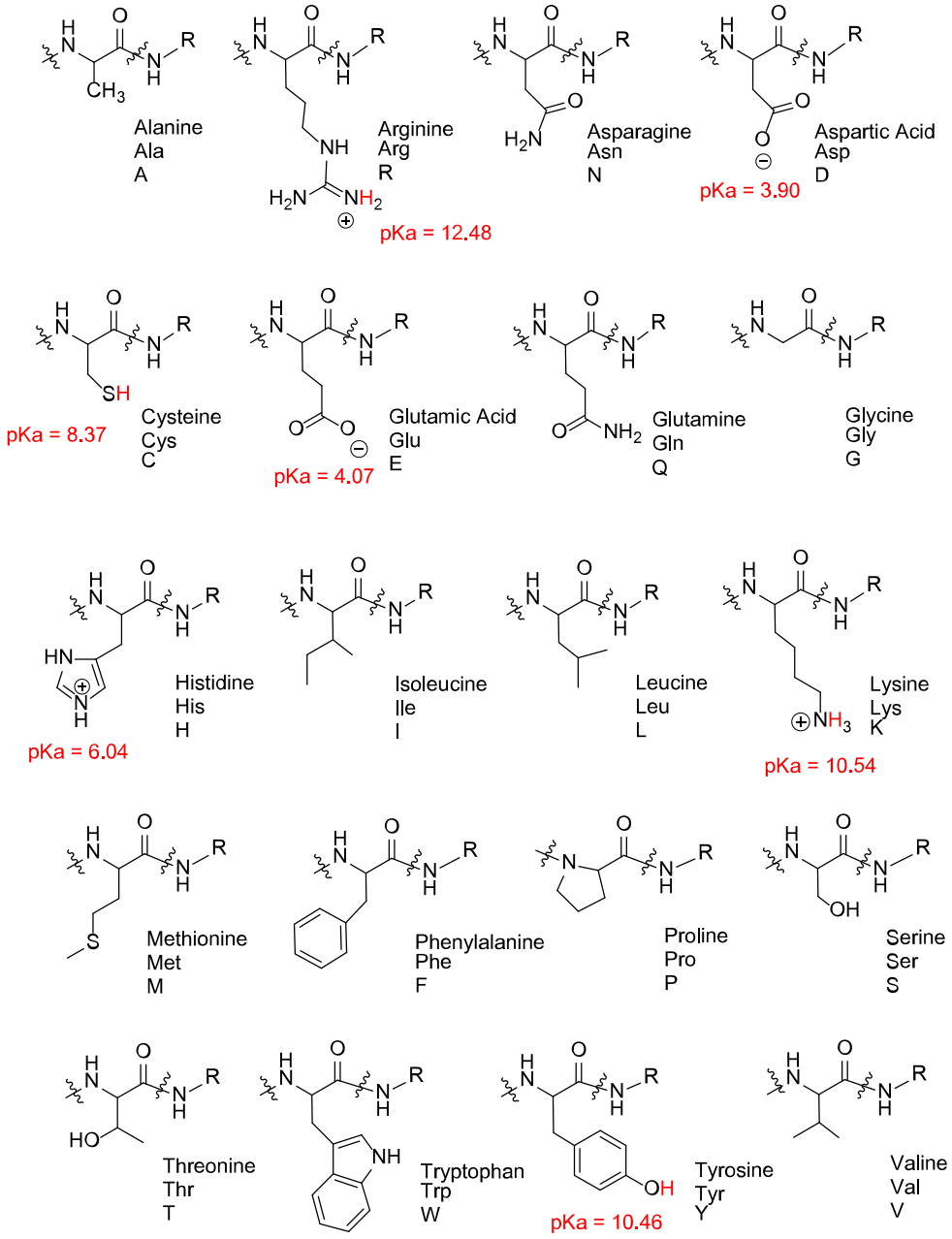


Figure 3.1.2: Side chains of naturally occurring amino acid embedded in a protein

Amino acids form polymers through a nucleophilic attack by the amino group of an amino acid at the electrophilic carbonyl carbon of the carboxyl group of another amino acid. The carboxyl group of the amino acid must first be activated to provide a better leaving group than OH-. The resulting link between the amino acids is an amide link, which biochemists call a peptide bond. In this reaction, water is released. In a reverse reaction, the peptide bond can be cleaved by water (hydrolysis). This is illustrated in Figure 3.1.3.

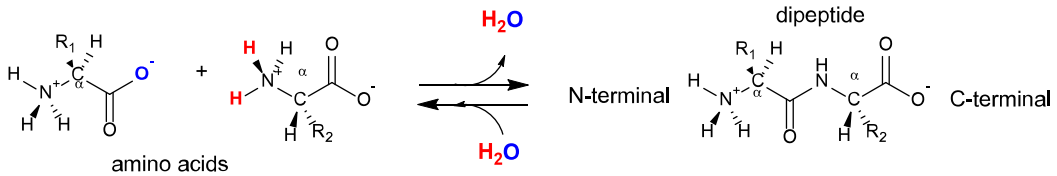


Figure 3.1.3: Amino Acids React to Form a Dipeptide

Proteins are polymers of twenty naturally occurring amino acids. In contrast, nucleic acids are polymers of just 4 different monomeric nucleotides. The sequence of a protein and its total length differentiate one protein from another. Just for an octapeptide, there are over 25 billion different possible arrangements of amino acids. Compare this to just 65536 oligonucleotides (4 different monomeric deoxynucleotides) of 8 monomeric units, an 8mer. Hence, the diversity of possible proteins is enormous.

The resulting structure is called a dipeptide when two amino acids form an amide link. Likewise, we can have tripeptides, tetrapeptides, and other polypeptides. At some point, when the structure is long enough, it is called a protein. The average molecular weight of proteins in yeast is about 50,000, with about 450 amino acids. The largest human protein might be titin, with a molecular weight of about 3 million (about 30,0000 amino acids). A new class of very small proteins (30 or fewer amino acids and perhaps better-named polypeptides) called smORFs (small open reading frames) have recently been discovered to have significant biological activity. These are encoded directly in the genome and are produced by the same processes that produce regular proteins (DNA transcription and RNA translation). They are not the result of selective cleavage of a larger protein into smaller peptide fragments.

Figure 3.1.4 shows several ways to represent the structure of a polypeptide or protein, each showing differing amounts of information. Note that the atoms in the side chains are denoted alpha, beta, gamma, delta, epsilon ...

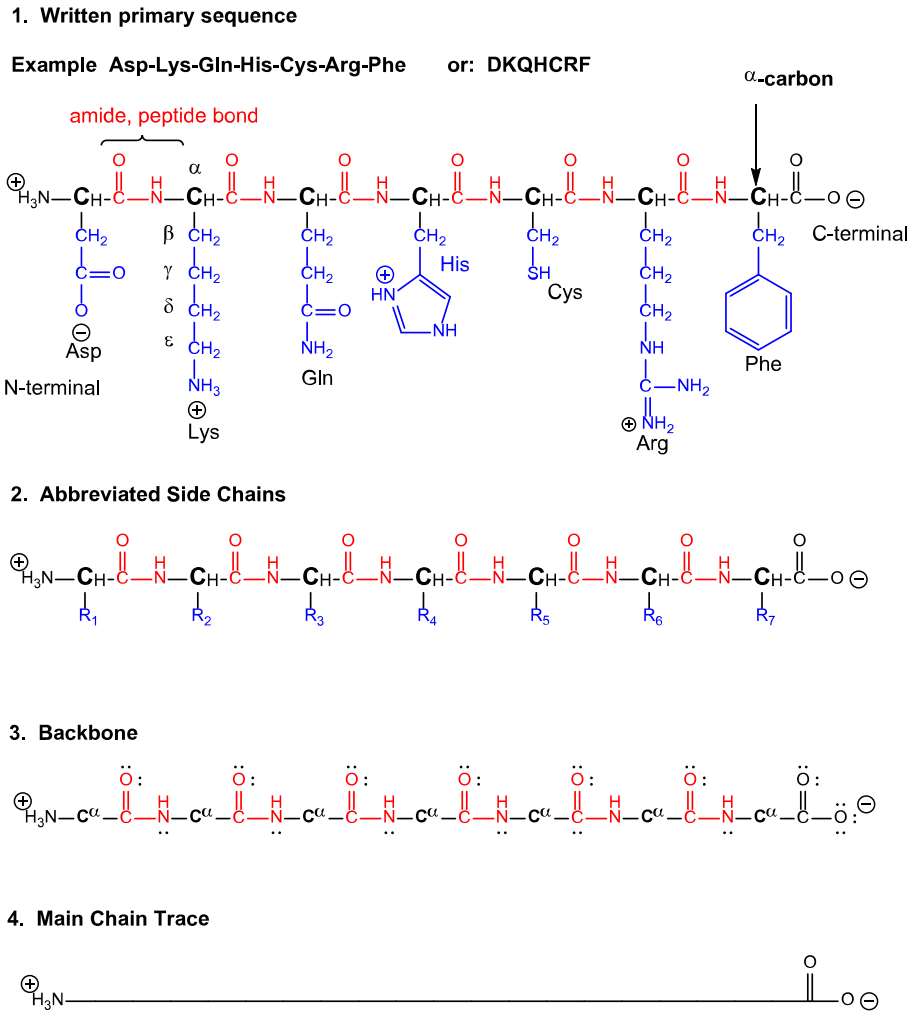


Figure 3.1.4: Different ways to represent the structure of a peptide/protein sequence.

3.1.3: Characteristics of Amino Acids

The different R-groups have different characteristics based on the nature of atoms incorporated into the functional groups. Some R-groups predominantly contain carbon and hydrogen and are very nonpolar or hydrophobic. Others contain polar uncharged functional groups such as alcohols, amides, and thiols. A few amino acids are basic (containing amine functional groups) or acidic (containing carboxylic acid functional groups). These amino acid side chains can form full charges and have ionic interactions.

Each amino acid can be abbreviated using a three-letter and a one-letter code. Figure 3.1.5 shows groupings of the amino acids based on their side chain properties.

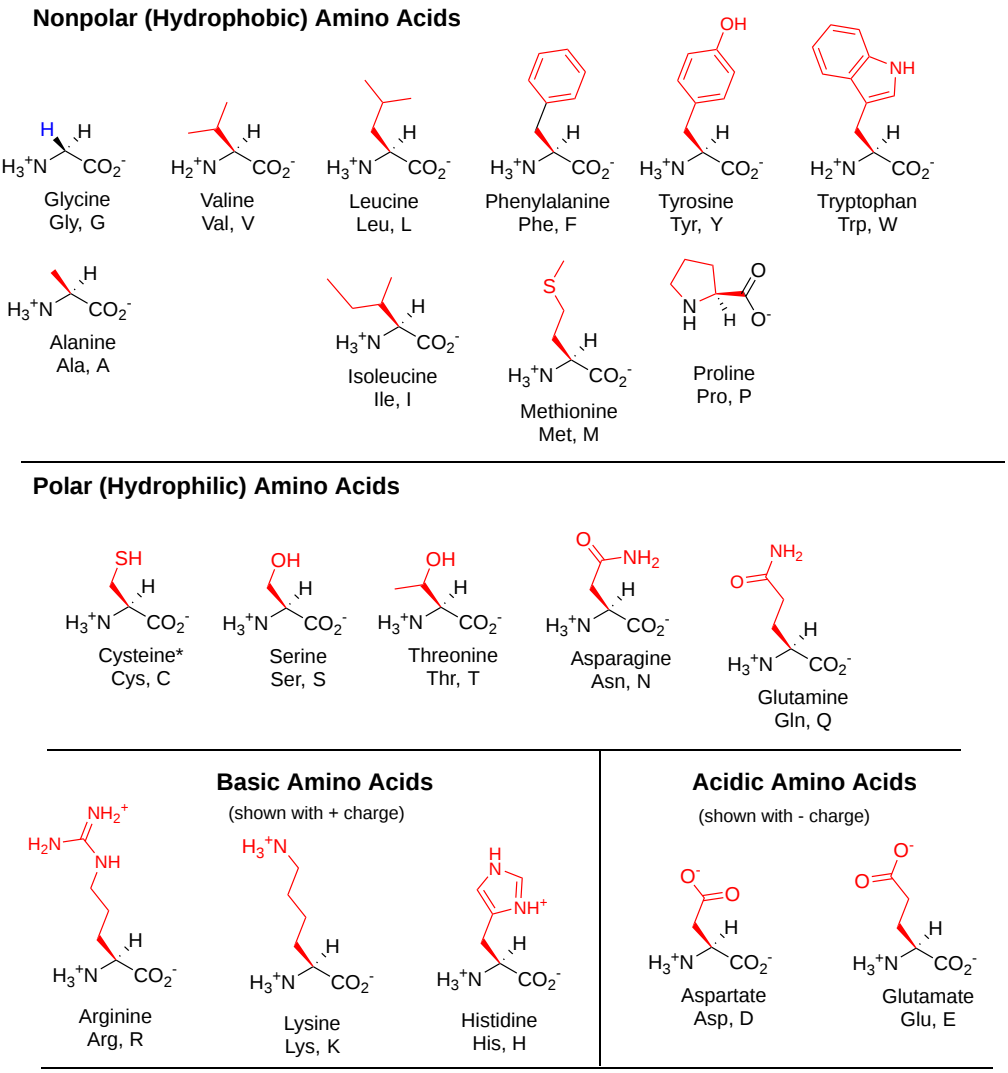


Figure 3.1.5: Structure of the 20 Alpha Amino Acids used in Protein Synthesis. R-groups are shown in **red**, connected to the alpha-carbon by a **red** wedge bond.

The above classification for a few amino acids is somewhat arbitrary, as described below. For example, cysteine is often buried in protein in a more hydrophobic environment.

3.1.4: Nonpolar (Hydrophobic) Amino Acids

The nonpolar amino acids can largely be subdivided into two more specific classes, the ***aliphatic*** amino acids and the ***aromatic*** amino acids. The ***aliphatic amino acids (glycine, alanine, valine, leucine, isoleucine, and proline)*** typically contain branched hydrocarbon chains, the simplest being glycine to the more complicated structures of leucine and valine. Proline is also classified as an aliphatic amino acid but contains special properties as the hydrocarbon chain has cyclized with the terminal amine, creating a unique 5-membered ring structure. As we will see in the next section covering primary structure, proline can significantly alter the 3-dimensional structure of the due to the structural rigidity of the ring structure when it is incorporated into the polypeptide chain and is commonly found in regions of the protein where folds or turns occur.

As their name implies, the ***aromatic amino acids (phenylalanine, tyrosine, and tryptophan)*** contain an aromatic functional group within their structure, making them largely nonpolar and hydrophobic due to their high carbon/hydrogen content. However, it should be noted that hydrophobicity and hydrophilicity represent a sliding scale, and each of the different amino acids can have

different physical and chemical properties depending on their structure. For example, the hydroxyl group in tyrosine increases its reactivity and solubility compared to phenylalanine.

Methionine, one of the sulfur-containing amino acids, is usually classified as a nonpolar, hydrophobic amino acid. The terminal methyl group is a thioether, which generally cannot form a permanent dipole within the molecule and retains low solubility.

3.1.5: Polar (Hydrophilic) Amino Acids

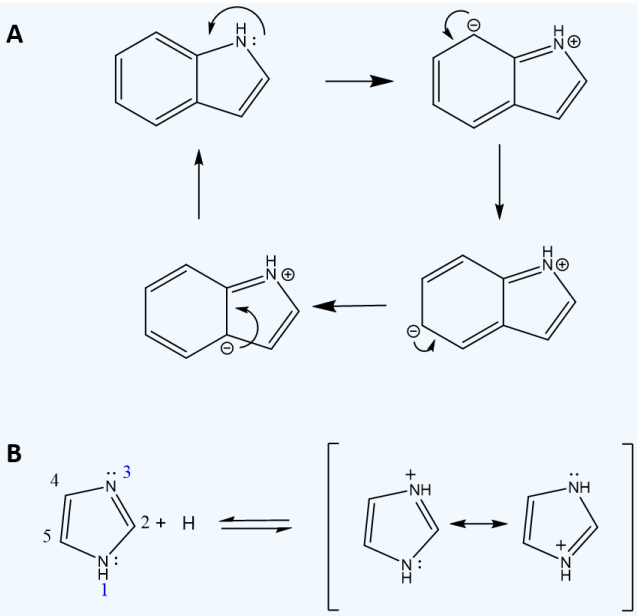
The polar, hydrophilic amino acids can be subdivided into three major classes: the polar uncharged-, the acidic-, and the basic-functional groups. Within the ***polar uncharged class,*** the side chains contain heteroatoms (O, S, or N) capable of forming permanent dipoles within the R-group. These include **the hydroxyl- and sulfhydryl-containing amino acids, serine, threonine, and cysteine**, and the amide-containing amino acids, **glutamine and asparagine.** Two amino acids, **glutamic acid (glutamate),** and **aspartic acid (aspartate)** constitute **the acidic amino acids** and contain side chains with carboxylic acid functional groups capable of fully ionizing in solution. The **basic amino acids, lysine, arginine, and histidine,** contain amine functional groups that can be protonated to carry a full charge.

Many amino acids with hydrophilic R-groups can participate within the ***active site*** of enzymes. An ***active site*** is the part of an enzyme that directly binds to a substrate and carries out a reaction. Protein-derived enzymes contain ***catalytic groups*** consisting of amino acid R-groups that promote the formation and degradation of bonds. The amino acids that play a significant role in the binding specificity of the active site are usually not adjacent to each other in the primary structure but form the active site as a result of folding in creating the tertiary structure, as you will see later in the chapter.

Example 3.1.1

Thought Question: Tryptophan contains an amine functional group. Why isn't tryptophan basic?

Answer: Tryptophan contains an indole ring structure with the amine functional group. However, due to the proximity of and electron-withdrawing nature of the aromatic ring structure, the lone pair of electrons on the nitrogen are unavailable to accept a proton. Instead, they form *pi*-bonds within several of the different resonance structures possible for the indole ring. Figure 2.3A shows four of the possible resonance structures for indole. Conversely, within the imidazole ring structure found in histidine, there are two nitrogen atoms, one of which is involved in the formation of resonance structures (Nitrogen #1 in Figure 2.3B) and cannot accept a proton, and the other (Nitrogen #3) that has a lone pair of electrons that is available to accept a proton.



Comparison of the Structural Availability of Lone Pair of Electrons on Nitrogen to Accept a Proton in the Indole and Imidazole Ring Structures. (A) Four resonance structures of the indole ring structure show that the lone pair of electrons on the nitrogen is involved in forming pi-bonds. (B) The imidazole ring structure has one nitrogen (1) that is involved in resonance structures (not shown) and is not available to accept a proton, while the second nitrogen (3) has a lone pair of electrons available to accept a proton, as shown.

Exercise 3.1.1

Work It Out on Your Own:

Given the example above, describe, using a chemical diagram, why the amide nitrogen atoms found in asparagine and glutamine are not basic.

Answer

The lone pair is delocalized into the peptide bond (different resonance structure), so it is unavailable for sharing.

Recent Updates: 5/5/24

3.1.6: Quantitative measures of amino acid polarity and hydrophobicity

There are quantitative ways to determine the relative polarity of an amino acid side chain, so it's not just a matter of visual inspection or a guess. Let's consider hydrophobicity or hydropathy scales. Most are based on the standard free energy of transfer of a side chain from water to a nonpolar solvent. Each amino acid side chain is given a number that varies from a negative to a positive value. The Kyte-Doolittle and the Hopp-Woods hydropathy scales are the two commonly used scales, as shown in Table 3.1.1 below.

Amino Acid	Kyte-Doolittle	Hopp-Woods
Alanine	1.8	-0.5
Arginine	-4.5	3.0
Asparagine	-3.5	0.2
Aspartic acid	-3.5	3.0

Cysteine	2.5	-1.0
Glutamine	-3.5	0.2
Glutamic acid	-3.5	3.0
Glycine	-0.4	0.0
Histidine	-3.2	-0.5
Isoleucine	4.5	-1.8
Leucine	3.8	-1.8
Lysine	-3.9	3.0
Methionine	1.9	-1.3
Phenylalanine	2.8	-2.5
Proline	-1.6	0.0
Serine	-0.8	0.3
Threonine	-0.7	-0.4
Tryptophan	-0.9	-3.4
Tyrosine	-1.3	-2.3
Valine	4.2	-1.5

Table 3.1.1: Kyte-Doolittle and Hopp-Woods hydrophobicity values

Note that the Hopp-Woods scale is more like a hydrophilicity scale since the more polar residues have more positive values. It was developed to locate likely antibody or other protein interaction sites on protein surfaces, which display more hydrophilic side chains.

Some discrepancies exist in which amino acid side chains are nonpolar between the Kyte-Doolittle values and Figure 3.1.5. The Kyte-Doolittle scale shows that glycine, the two large aromatics tyrosine and tryptophan, and proline are more polar than nonpolar and that cysteine is quite nonpolar.

As we will see in subsequent sections, a continuous stretch of amino acids found to have a high average hydrophobicity (low hydrophilicity) is probably buried in the interior of a protein away from the aqueous environment. Conversely, a continuous stretch with low hydrophobicity (high hydrophilicity) is likely buried in a protein or a membrane bilayer. Consider the example of the water-soluble bovine alpha-chymotrypsinogen, a 245 amino acid protein, whose sequence is shown below in single letter code.

1 CGVPAIQPVLSGLSRIVNGEEAVPGSWPWQVSLQDKTGFHFCGGSLINENWVVTAAHCGV
61 TTSDVVVAGEFDQGSSSEKIQKLKIAKVFKNSKYNSLTINNDITLLKLSTAASFSQTVSA
121 VCLPSASDDFAAGTTCVTTGWGLTRYTNANTPDRLQQASLPLLSNTNCKKYWGTKIKDAM
181 ICAGASGVSSCMGDSGGPLVCKKNGAWTLVGIVSWGSSTCSTSTPGVYARVTALVNWVQQ
241 TLAAN

Use the ExPasy Prot Scale server to produce hydrophobicity plots of the protein bovine α-chymotrypsinogen. Input the Uniprot number **P00766** for the protein or the sequence into the appropriate boxes. Select a length of continuous amino acids (called a window) of 7, and the program will calculate an average hydrophobicity for the "window." The window slides down the linear sequence, and a new value is calculated to determine a series of values for the entire sequence. Hydropathy plots (average score for the midpoint amino acid in the window) for chymotrypsinogen (window of seven consecutive residues) are shown in Figure 3.1.6 below. The Kyte-Doolittle scale (+ is hydrophobic) shows many stretches with high average values. The amino acids at those

positions are likely buried in the protein's interior. The Hoop-Woods value (+ is hydrophilic) shows stretches with high average values. These amino acids are likely on the surface exposed to water. The two plots are complementary to each other.

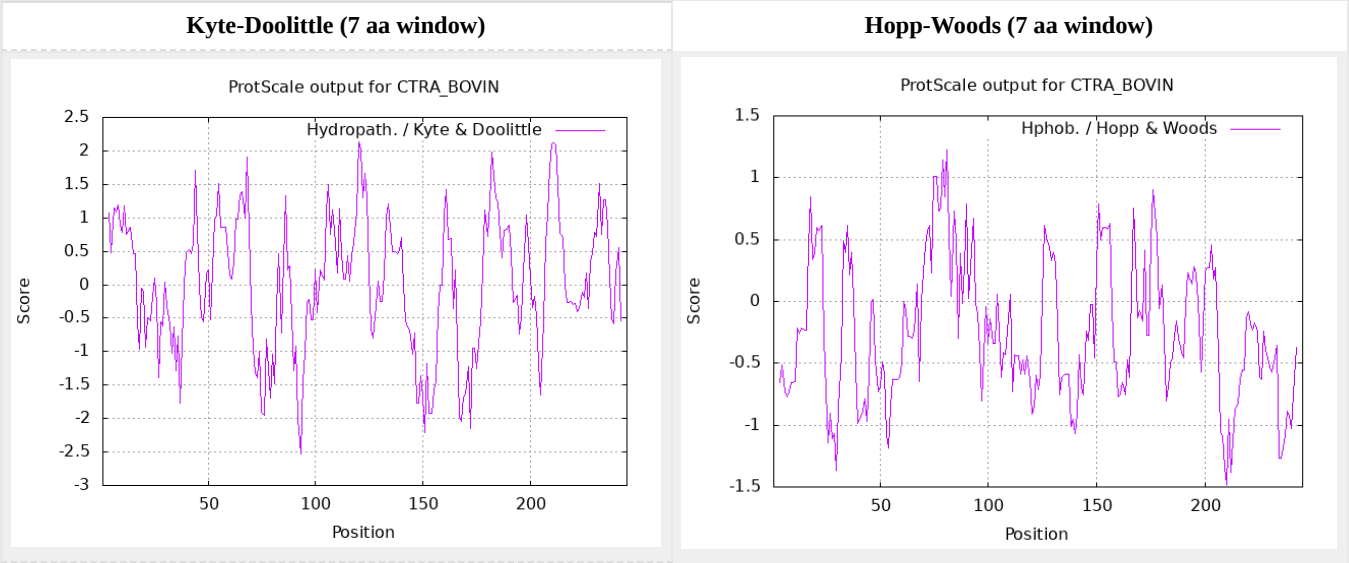


Figure 3.1.6: Kyte-Doolittle and Hopp-Woods plots for bovine α-chymotrypsinogen

3.1.7: Amino Acid Stereochemistry

The amino acids are all ***chiral*** except glycine, whose side chain is H. A ***chiral molecule*** is not superimposable with its mirror image. Like left and right hands that have a thumb and fingers in the same order but are mirror images and not the same, chiral molecules have the same things attached in the same order but are mirror images and not the same. The mirror image versions of chiral molecules have physical properties that are nearly identical to one another, making it very difficult to tell them apart from one another or to separate them. Because of this, they are given a special stereoisomer name called ***enantiomers,*** and the compounds themselves are given the same name! These molecules differ in how they rotate plane-polarized light and react with and interact with biological molecules. Molecules that rotate light in the right-handed direction are called dextrorotary and are given a small "d" letter designation. Molecules that rotate light in the left-handed direction are called levorotary and are given a small "l" letter designation to distinguish one enantiomer from the other. Biochemists also use the older nomenclature of large "L" and "D" to characterize the 3D stereochemistry of the amino acids. All naturally occurring proteins from all living organisms consist of L amino acids based on their structural similarities to L-glyceraldehyde.

Again, the d- and l-designations are specific terms for how a molecule rotates plane-polarized light. It does **not** denote the absolute stereo configuration of a molecule. An **absolute configuration** refers to the spatial arrangement of the atoms of a chiral molecular entity (or group) and its modern stereochemical description, e.g., **R** or **S**, referring to **Rectus** or **Sinister**, respectively. Absolute configurations for a chiral molecule (in pure form) are often determined by X-ray crystallography. Alternative techniques are optical rotatory dispersion, vibrational circular dichroism, chiral shift reagents in proton NMR, and Coulomb explosion imaging. When the absolute configuration is known, the assignment of *R* or *S* is based on the **Cahn–Ingold–Prelog priority rules**. The absolute stereochemistry is related to L-glyceraldehyde, as shown below in Figure 3.1.6.

All naturally occurring amino acids in proteins are L, which corresponds to the S isomer, except cysteine. As shown in the bottom left of Figure 3.1.7 below, the absolute configuration of the amino acids can be shown with the H pointed to the rear, the COOH groups pointing out to the left, the R group to the right, and the NH_3 group upwards. You can remember this with the mnemonic "CORN".

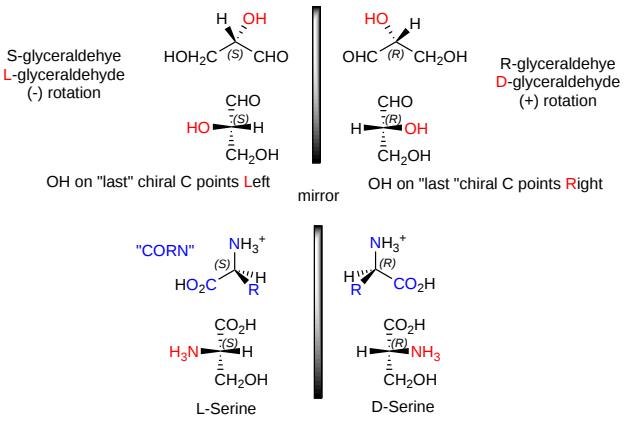


Figure 3.1.7: Stereochemistry of amino acids

Why does Biochemistry still use D and L for sugars and amino acids? This explanation (taken from a website that may not be available anymore, so no reference is available) seems reasonable.

"In addition, however, chemists often need to define a configuration unambiguously in the absence of any reference compound, and for this purpose, the alternative (R,S) system is ideal, as it uses priority rules to specify configurations. These rules sometimes produce absurd results when applied to biochemical molecules. For example, as we have seen, all of the common amino acids are L because they all have the same structure, including the position of the R group if we just write the R group as R. However, they do not all have the same configuration in the (R, S) system: L-cysteine is also (R)-cysteine. Still, all the other L-amino acids are (S), but this reflects the human decision to give a sulfur atom a higher priority than a carbon atom and does not reflect a real difference in configuration. Worse problems can sometimes arise in substitution reactions: sometimes, inversion of configuration can result in no change in the (R) or (S) prefix, and sometimes, retention of configuration can result in a change of prefix.

It follows that it is not just conservatism or failure to understand the (R,S) system that causes biochemists to continue with D and L: it is just that the DL system fulfills their needs much better. As mentioned, chemists also use D and L when appropriate to their needs. The “explanation” given above of why the (R, S) system is little used in biochemistry is thus almost the exact opposite of reality. This system is the only practical way of unambiguously representing the stereochemistry of complicated molecules with several asymmetric centers, but it is inconvenient with regular series of molecules like amino acids and simple sugars."

If you are told to draw the correct stereochemistry of a molecule with one chiral C (S isomer, for example) and are given the substituents, you could do so easily following the R, S priority rules. However, how would you draw the correct isomer for the L isomer of the amino acid alanine? You couldn't do it without prior knowledge of the absolute configuration of the related molecule, L glyceraldehyde, or unless you remembered the anagram CORN. However, this disadvantage is more than made up for because different L amino acids with the same absolute stereochemistry might be labeled R or S, making this nomenclature unappealing to biochemists.

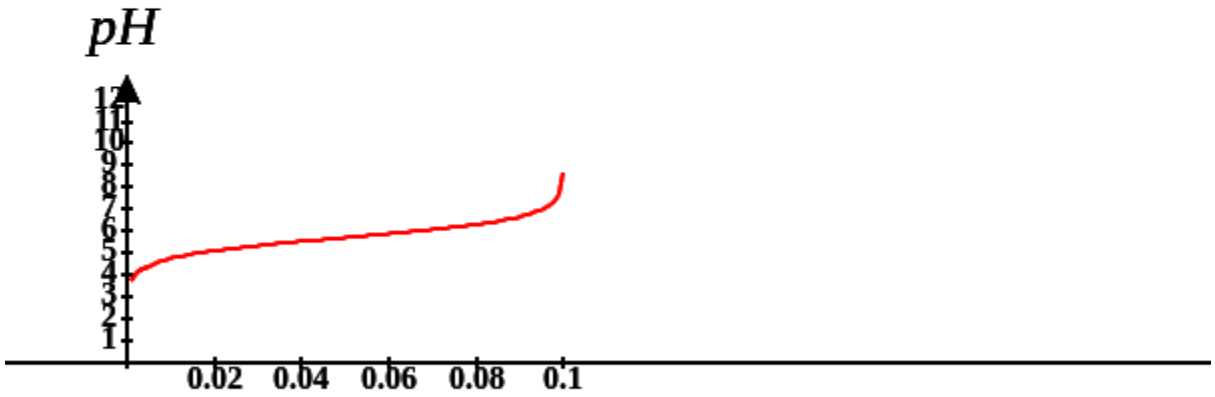
3.1.8: Amino Acid Charges

Monomeric amino acids have an alpha-amino group and a carboxyl group, both of which may be protonated or deprotonated, and an R group, some of which may be protonated or deprotonated. When protonated, the amino group has a +1 charge, and the carboxyl group has a zero charge. When deprotonated, the amino group has no charge, while the carboxyl group has a -1 charge. The R groups that can be protonated/deprotonated include Lys, Arg, and His, which have a + 1 charge when protonated, and Glu and Asp (carboxylic acids), Tyr and Ser (alcohols) and Cys (thiol), which have 0 charges when protonated. Of course, when the amino acids are linked by peptide bonds (amide link), the alpha N and the carboxyl C are in an amide link and are not charged.

However, the amino group of the N-terminal amino acid and the carboxyl group of the C-terminal amino acid of a protein may be charged. The Henderson-Hasselbalch equation allows us to determine the charge state of any ionizable group knowing the pKa of the group. Write each functional group capable of being deprotonated as an acid, HA, and the deprotonated form as A. The charge of HA and A will be determined by the functional group and the Henderson-Hasselbalch equation from Chapter 2.2.

$$\mathrm{pH} = \mathrm{p}K_\mathrm{a} + \log \frac{[\mathrm{A}^-]}{[\mathrm{HA}]} \tag{3.1.1}$$

The titration curve for a single ionizable acid with different pKa values is shown below.



At the curve's inflection point, pH = pKa, the system is most resistant to changes in pH when adding either acid or base. At this pH, [HA]=[A^-].

The properties of a protein will be determined partly by whether the side chain functional groups, the N terminal and the C terminal, are charged or not. The HH equation tells us that this will depend on the pH and the pKa of the functional group.

- If the pH is 2 units below the pKa, the HH equation becomes -2 = log A/HA, or .01 = A/HA. This means the functional group will be about 99% protonated (with either 0 or +1 charge, depending on the functional group).
- If the pH is 2 units above the pKa, the HH equation becomes 2 = log A/HA, or 100 = A/HA. Therefore, the functional group will be 99% deprotonated.
- If the pH = pKa, the HH equation becomes 0 = log A/HA or 1 = A/HA. Therefore, the functional group will be 50% deprotonated.

From these simple examples, we have derived the +2 rule. This rule is used to quickly determine protonation, and hence charge state, and is extremely important to know (and easy to derive). Titration curves for Gly (no ionizable) side chain, Glu (carboxylic acid side chain), and Lys (amine side chain) are shown in Figure 3.1.8. You should be able to associate various sections of these curves with the titration of specific ionizable groups in the amino acids.

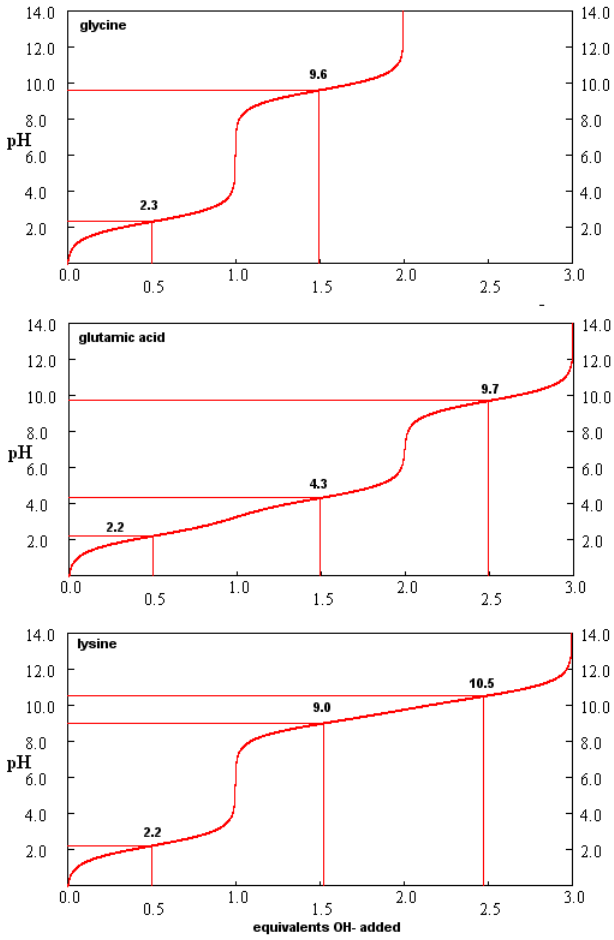


Figure 3.1.8: Titration curves for Gly, Glu, and Lys

New 5/16/23: Download this Excel spreadsheet for Titration Curves for a Triprotic Acid. It has adjustable scroll bars to change pK_a values.

3.1.9: Buffer Review

The Henderson-Hasselbalch equation is also useful in calculating the composition of buffer solutions. Remember that buffer solutions are composed of a weak acid and its conjugate base. Consider the equilibrium for a weak acid, like acetic acid, and its conjugate base, acetate:

$$CH_3CO_2H + H_2O \rightleftharpoons H_3O^+ + CH_3CO_2^-$$

If the buffer solution contains equal concentrations of acetic acid and acetate, the pH of the solution is:

or pH = pKa + log [A]/[HA] = 4.7 + log 1 = 4.7

A look at the titration curve for the carboxyl group of Gly (see above) shows that when the pH = pKa, the slope of the curve (i.e. the change in pH on addition of base or acid) is at a minimum. As a general rule of thumb, buffer solutions can be made for a weak acid/base in the range of +/- 1 pH unit from the pKa of the weak acids. At the pH = pKa, the buffer solution best resists adding either an acid or a base and has its greatest buffering ability. The weak acid can react with the added strong base to form the weak conjugate base, and the conjugate base can react with added strong acid to form the weak acid (as shown below), so pH changes with the addition of strong acid and base are minimized.

- addition of a strong base produces a weak conjugate base: $CH_3CO_2H + OH^- \leftrightarrow CH_3CO_2^- + H_2O$
- addition of strong acid produces weak acid: $H_3O^+ + CH_3CO_2 \rightarrow CH_3CO_2H + H_2O$

There are two simple ways to make a buffered solution. Consider an acetic acid/acetate buffer solution.

- make equal molar solutions of acetic acid and sodium acetate and mix them, monitoring pH with a pH meter, until the desired pH is reached (+/- 1 unit from the pKa).
- take an acetic acid solution and add NaOH at substoichiometric amounts until the desired pH is reached (+/- 1 unit from the pKa). In this method, you are forming the conjugate base, acetate, with the addition of NaOH:

$$CH_3CO_2H + OH^- \rightarrow CH_3CO_2^- + H_2O$$

- Buffers for pH control: Recipes based on pKas for acids, temperature, and ionic strength

3.1.10: Isoelectric Point

What happens if you have many ionizable groups in a single molecule, as is the case with a polypeptide or a protein? Consider a protein. At a pH of 2, all ionizable groups would be protonated, and the overall charge of the protein would be positive. (Remember, when carboxylic acid side chains are protonated, their net charge is 0.) As the pH is increased, the most acidic groups will start to deprotonate, and the net charge will become less positive. At high pH, all the ionizable groups will become deprotonated in the strong base, and the overall charge of the protein will be negative. At some pH, then, the net charge will be 0. This pH is called the isoelectric point (pI). The pI can be determined by averaging the pKa values of the two closest groups straddling the pI. One of the online problems will address this in more detail.

Remember that pKa is a measure of the equilibrium constant for the reaction. And, of course, you remember that $\Delta G^o = -RT \ln Keq$. Therefore, pKa is independent of concentration and depends only on the intrinsic stability of reactants with respect to the products. This is true only AT A GIVEN SET OF CONDITIONS, SUCH AS T, P, AND SOLVENT CONDITIONS.

Consider, for example, acetic acid, which in aqueous solution has a pKa of about 4.7. It is a weak acid, which dissociates only slightly to form H+ (in water the hydronium ion, H_3O^+, is formed) and acetate (Ac^-). These ions are moderately stable in water but reassociate readily to form the starting product. The pKa of acetic acid in 80% ethanol is 6.87. This can be accounted for by the decrease in stability of the charged products, which are less shielded from each other by the less polar ethanol. Ethanol has a lower dielectric constant than water. The pKa increases to 10.32 in 100% ethanol and a whopping 130 in air!

Because amino acids are zwitterions, and several also contain the potential for ionization within their R-groups, their charge state *in vivo*, and thus, their reactivity can vary depending on the pH, temperature, and solvation status of the local microenvironment in which they are located. Table 3.1.2 shows the standard pK_a values for the amino acids and can be used to predict their ionization/charge status and their resulting peptides/proteins.

pK$_a$ Values for Common Alpha Amino Acids				
Amino Acid Type	Amino Acid	α-COOH	α-NH_3^+	RH or RH^+
Hydrophobic: Aliphatic	Glycine	2.34	9.60	
	Alanine	2.34	9.69	
	Valine	2.32	9.62	
	Leucine	2.36	9.68	
	Isoleucine	2.36	9.68	
	Proline	1.99	10.6	
	Methionine	2.28	9.21	
Hydrophobic: Aromatic	Phenylalanine	1.83	9.13	
	Tyrosine	2.2	9.11	10.07
	Tryptophan	2.38	9.39	
Hydrophilic: Polar Uncharged	Serine	2.21	9.15	
	Threonine	2.63	10.43	
	Cysteine	1.71	10.78	8.33
	Asparagine	2.02	8.8	
	Glutamine	2.17	9.13	
Hydrophilic: Acidic	Aspartic Acid	2.09	9.82	3.86
	Glutamic Acid	2.19	9.67	4.25
Hydrophilic: Basic	Arginine	2.17	9.04	12.48
	Histidine	1.82	9.17	6.00
	Lysine	2.18	8.95	10.53

Table 3.1.2: Summary of pKas of amino acids

However, it should be noted that the solvation status in the microenvironment of an amino acid can alter the relative pK_a values of these functional groups and provide unique reactive properties within the active sites of enzymes. A more in-depth discussion of the effects of desolvation will be given in Chapter 6, discussing enzyme reaction mechanisms.

- *Printable Version of pKa Values*
- Expasy pI and molecular weight calculator for any protein sequence

3.1.11: Introduction to Amino Acid Reactivity

You should be able to identify which side chains contain H bond donors and acceptors. Likewise, some are acids and bases. You should know the approximate pKas of the side chains and the N and C terminal groups. Three of the amino acid side chains (Trp, Tyr, and Phe) contribute significantly to the UV absorption of a protein at 280 nm. This section will deal predominantly with the chemical reactivity of the side chains, which is important in understanding the properties of the proteins. Many of the side chains are nucleophiles. Nucleophilicity measures how rapidly molecules with lone pairs of electrons can react in nucleophilic substitution reactions. It correlates with basicity, which measures the extent to which a molecule with lone pairs can react with an acid (Bronsted or Lewis). The properties of the atom that holds the lone pair are important in determining both nucleophilicity and basicity. In both cases, the atom must be willing to share its unbonded electron pair. If the atoms holding the nonbonded pair are more electronegative, they will be less likely to share electrons, and that molecule will be a poorer nucleophile (nu:) and weaker base. Using these ideas, it should be clear that RNH_2 is a better nucleophile than ROH, OH^- is better than H_2O, and RSH is better than H_2O. In the latter case, S is bigger, and its electron cloud is more polarizable - hence, it is more reactive. The important side chain nucleophiles (in order from most to least nucleophilic) are Cys (RSH, pKa 8.5-9.5), His (pKa 6-7), Lys (pKa 10.5) and Ser (ROH, pKa 13). The side chain of serine is generally no more reactive than ethanol. It is a potent nucleophile in a certain class of proteins (proteases, for example) when deprotonated. The amino group of lysine is a potent nucleophile only when deprotonated.

An understanding of the chemical reactivity of the various R group side chains of the amino acids in a protein is important since chemical reagents that react specifically with a given amino acid side chain can be used to:

- identify the presence of the amino acids in unknown proteins or
- determine if a given amino acid is critical for the structure or function of the protein. For example, suppose a reagent that covalently interacts with only Lys is found to inhibit the protein's function. A lysine might be considered important in the protein's catalytic activity in that case.

Figure 3.1.9 summarizes nucleophilic addition and substitution at carbonyl carbons.

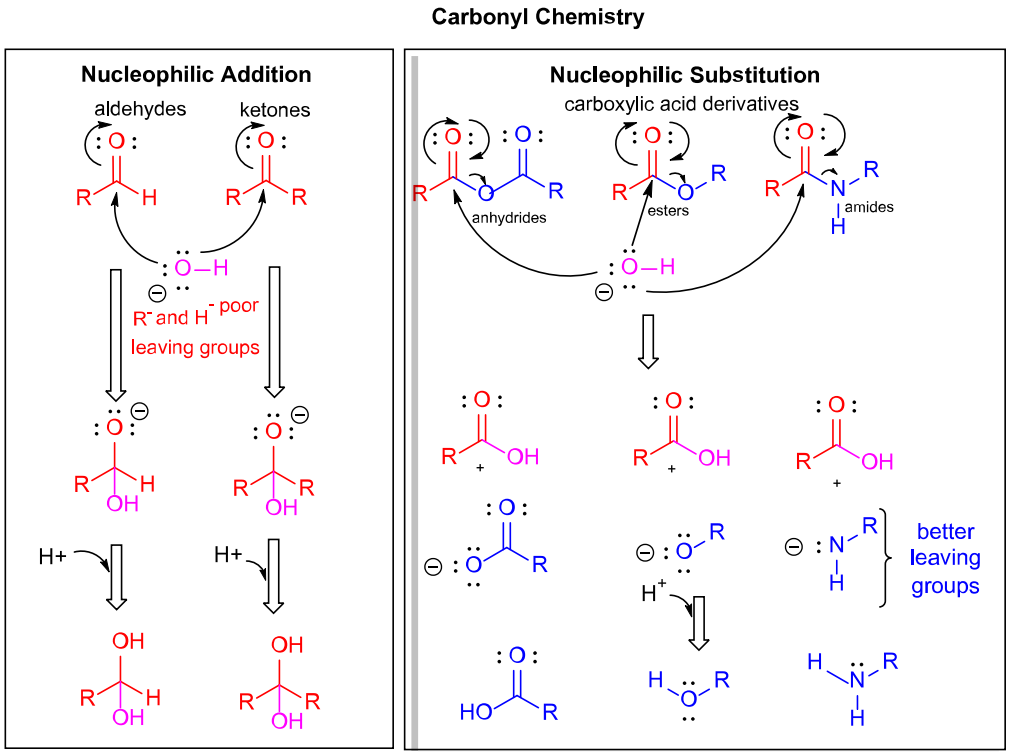


Figure 3.1.9: A review summary of the chemistry of aldehydes, ketones and carboxylic acid derivatives

The rest of the section will summarize the chemistry of the side chains of reactive amino acids. Historically, the function of a given amino acid in a protein has been studied by reacting it with side chain-specific chemical modifying agents. In addition, some side chains are covalently modified after they are synthesized in vivo (post-translational modification—see below).

3.1.12: Reactions of Lysine

Figure 3.1.10 shows the reaction of lysine with anhydrides and ethylacetimidate.

- reacts with anhydrides in a nucleophilic substitution reaction (acylation).
- reacts reversibly with methylmaleic anhydride (also called citraconic anhydride) in a nucleophilic substitution reaction.
- reacts with high specificity and yields toward ethylacetimidate in a nucleophilic substitution reaction (ethylacetimidate is like ethylacetate only with an imido group replacing the carbonyl oxygen). Ethanol leaves as the amidino group forms. (has two N - i.e. din - attached to the C)

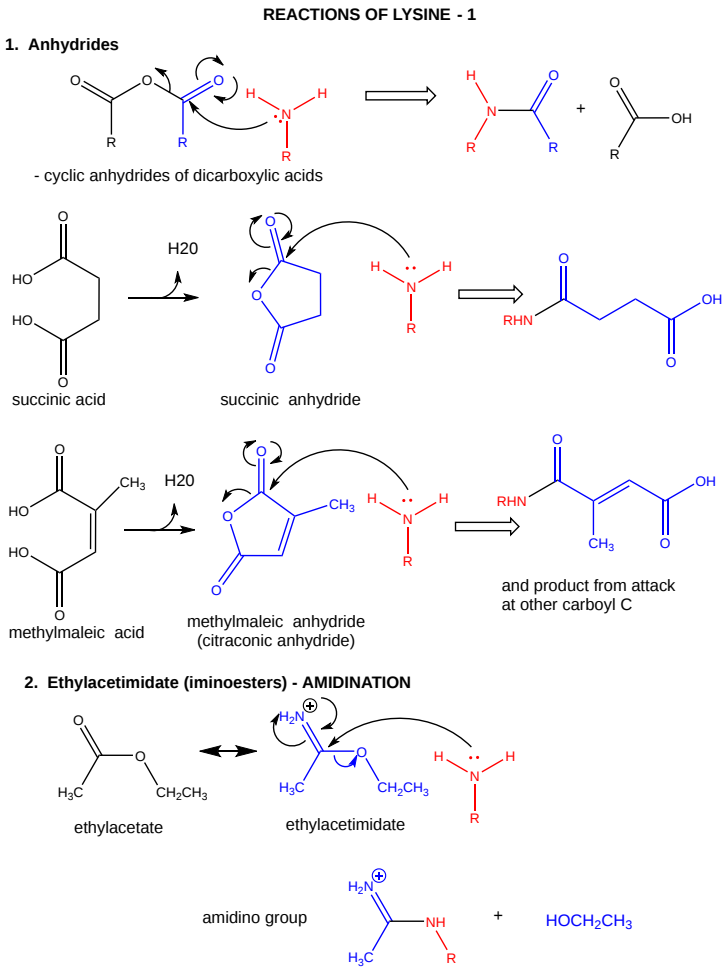


Figure 3.1.10: Reaction of lysine with anhydrides and ethylacetimidate.

Figure 3.1.11 shows a second set of common reactions of lysine, including those used to attach a chromophore or a fluorescent label to the side chain.

- reacts with O-methylisourea in a nucleophilic substitution reaction. with the expulsion of methanol to form a guanidino group (has 3 N attached to C, nidi)
- reacts with fluorodinitrobenzene (FDNB or Sanger's reagent) or trinitrobenzenesulfonate (TNBS, as we saw with the reaction with phosphatidylethanolamine) in a nucleophilic aromatic substitution reaction to form 2,4-DNP-lysine or TNB-lysine.
- reacts with Dimethylaminonapthelenesulfonylchloride (Dansyl Chloride) in a nucleophilic substitution reaction.

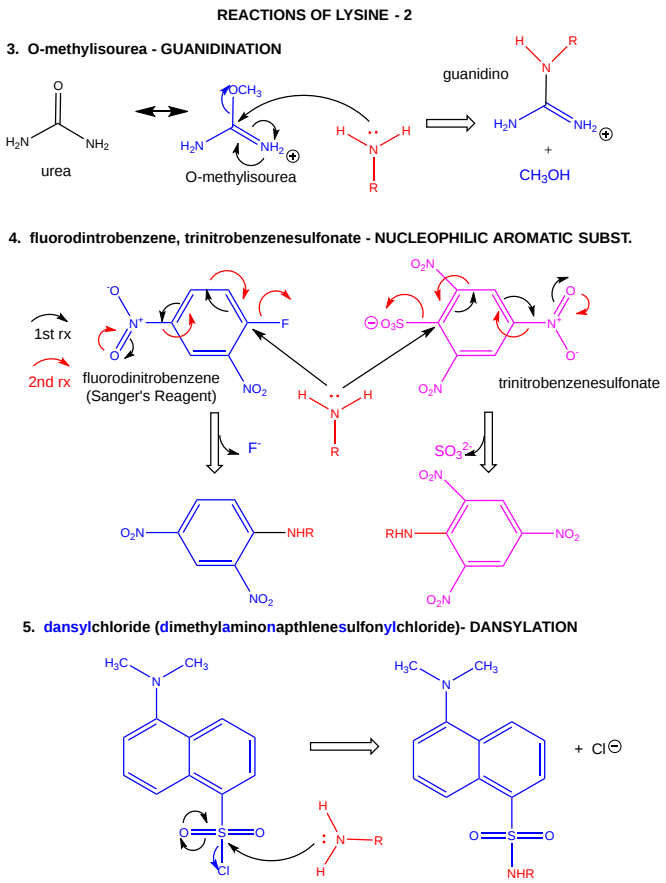


Figure 3.1.11: Reaction of lysine with O-methylisourea, chromophores, and fluorophores

Figure 3.1.12 shows a final common reaction we will encounter: the formation of an imine or Schiff base on the reaction of lysine with an aldehyde or ketone.

- reacts with high specificity toward aldehydes to form imines (Schiff bases), which can be reduced with sodium borohydride or cyanoborohydride to form a secondary amine.

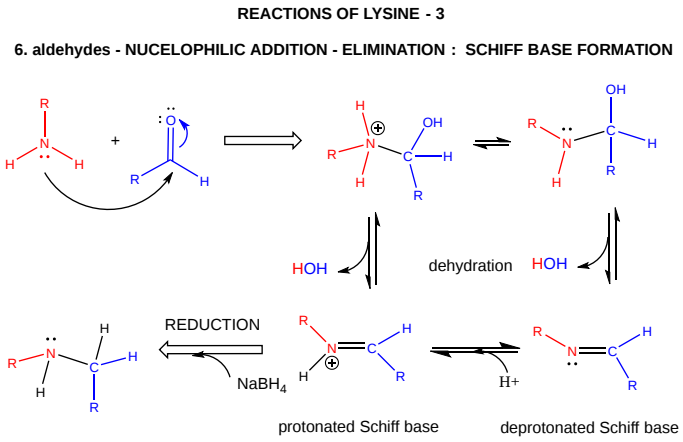


Figure 3.1.12: Reaction of lysine with an aldehyde or ketone to form a Schiff base

3.1.13: Reactions of Cysteine

Cysteine is a potent nucleophile often linked to another Cys to form a covalent disulfide bond.

Figure 3.1.13 shows common reagents used in the lab to label free Cys side chains. These reagents are used to alter Cys side chains to determine if they have functional significance in a protein (such as an active nucleophile in an enzyme-catalyzed reaction).

- reacts with iodoacetic acid in an S_N2 reaction, adding a carboxymethyl group to the S.

- reacts with iodoacetamide in an S_N2 reaction, adding a carboxyamidomethyl group to S.
- reacts with N-ethylmaleimide in an addition reaction to the double bond

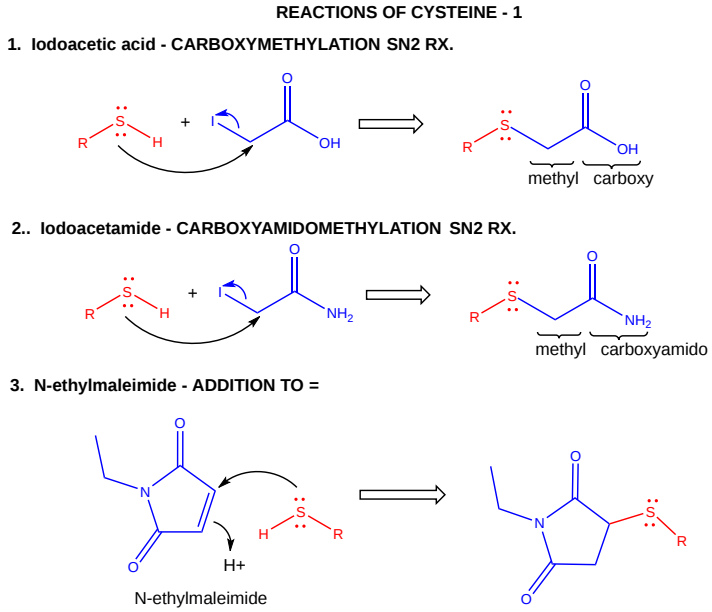


Figure 3.1.13: Common labeling reactions of cysteine

Sulfur is directly below oxygen in the periodic table, and, in analogy to water, sulfur-containing amino acids are found in different redox states, as illustrated in Figure 3.1.14.

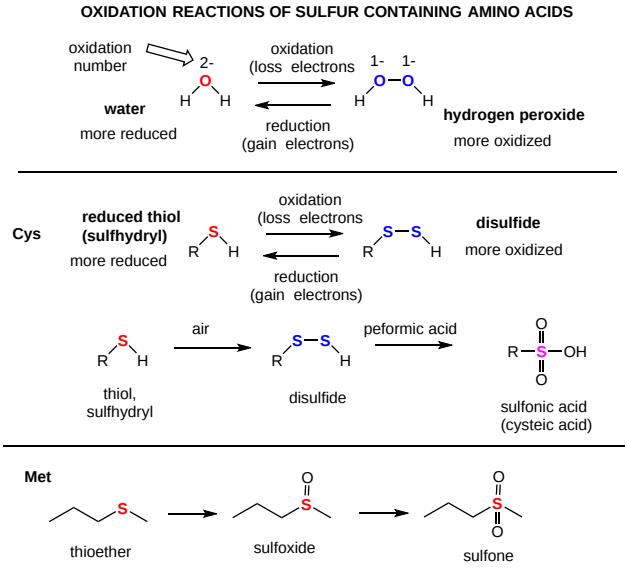


Figure 3.1.14: Oxidation states of sulfur

3.1.14: Cystine Chemistry

Two cysteine side chains can covalently interact in a protein to produce a disulfide (RS-SR) named cystine. Just as HOOH (hydrogen peroxide) is more oxidized than HOH (O in H_2O_2 has an oxidation number of 1- while the O in H_2O has an oxidation number of -2), RSSR is the oxidized form (S oxidation number -1), and RSH is the reduced form (S oxidation number -2) of thiols. Their oxidation numbers are analogous since O and S are both in Group 6 of the periodic table and are more electronegative than C.

Cystine can react with a free sulfhydryl (RSH) in a thermodynamically non-challenging disulfide **exchange** reaction, which when conducted with excess free sulfhydryls, results in the reduction of cystine in the protein, as shown in Figure 3.1.15.

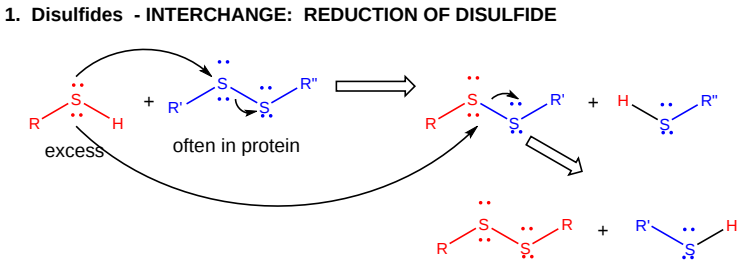


Figure 3.1.15: Disulfide interchange and reduction of protein disulfides

This reaction is often used in the lab to quantify the amount of free cysteine side chains in a protein using Ellman's reagent, as shown in Figure 3.1.16.

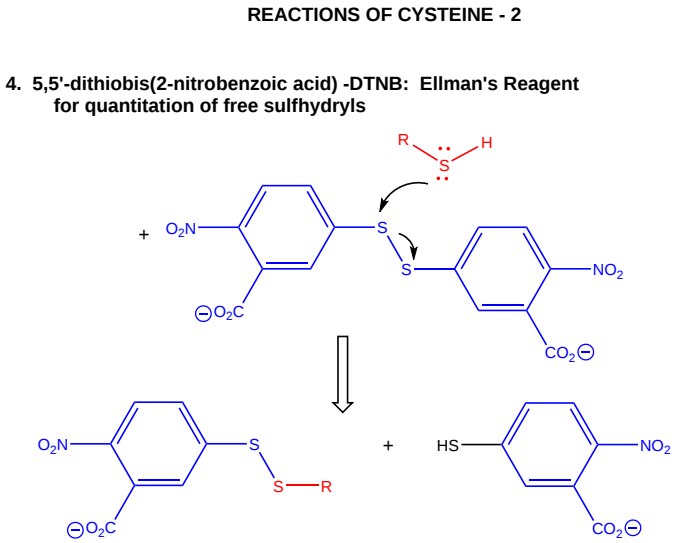


Figure 3.1.16: Reaction of free cysteine with Ellman's reagent.

The 2-nitro-5-thiobennzoic acid anion leaving group absorbs at 412 nm, making quantitation easy. However, unless the protein is unfolded to expose all the cysteines, only surface and not buried free cysteines will be labeled.

When a protein folds, two Cys side chains might approach each other and form an intra-chain disulfide bond. Likewise, two Cys side chains on separate proteins might approach each other and form an inter-chain disulfide. For analysis of the protein structure, disulfides are typically cleaved, and then the chains are separated The disulfides can be cleaved by reducing agents such as beta-mercaptoethanol, dithiothreitol, tris (2-carboxyethyl) phosphine (TCEP), or by oxidizing agents like performic acid, which further oxidizes the disulfide to separate cysteic acids. Three common reagents used in disulfide cleavage reactions in the lab are shown in Figure 3.1.17.

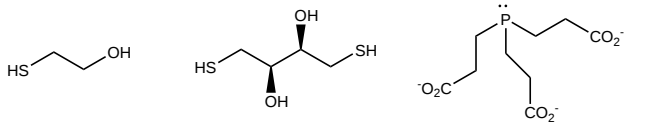


Figure 3.1.17: Three common disulfide cleaving (reducing) agents used in the lab

The reaction for beta-mercaptoethanol (BME) and performic acid is shown in Figure 3.1.18 below.

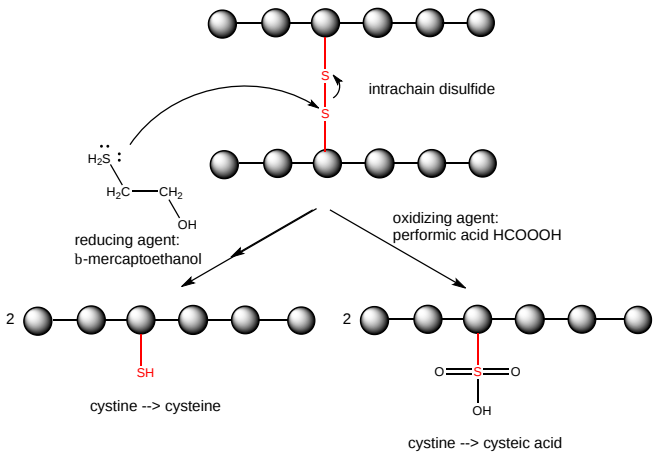


Figure 3.1.18: Cleave of intrachain cystine disulfide bonds in proteins by beta-mercaptoethanol and performic acid

Figure 3.1.19 shows the reaction for dithiothreitol (DTT). Note that it forms a stable cyclohexane-like ring, which favors this reaction thermodynamically. It does not require as much excess DTT as the reaction with BME.

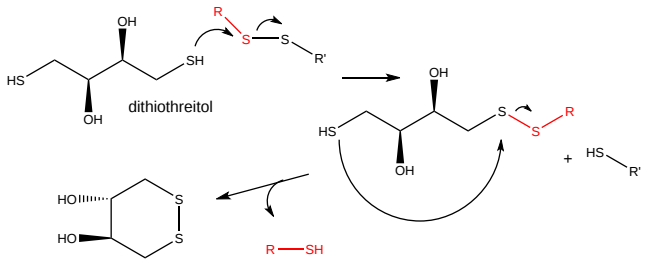


Figure 3.1.19: Cleave of disulfides with dithiothreitol

The reaction with tris (2-carboxyethyl) phosphine (TCEP) is not a disulfide interchange reaction, as is shown in Figure 3.1.20

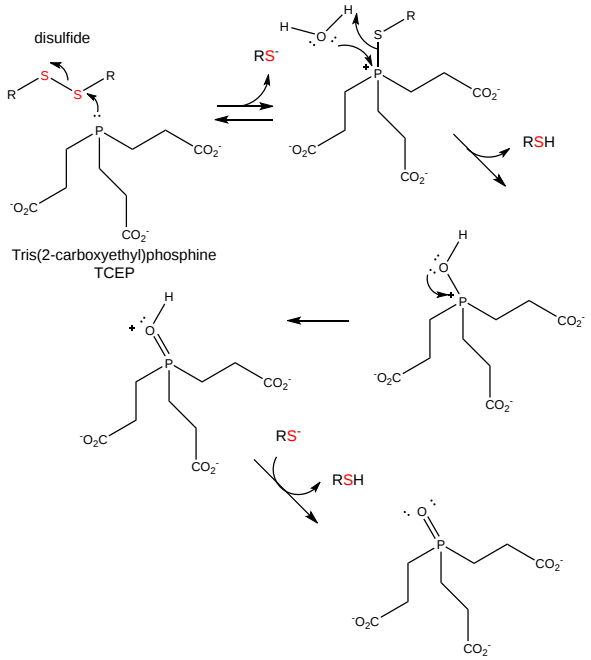


Figure 3.1.20: Reaction of TCEP with disulfides

Cells maintain a reducing environment using many "reducing" agents, such as the tripeptide gamma-Glu-Cys-Gly (glutathione). Hence intracellular proteins usually do **not** contain disulfides, which are abundant in extracellular proteins (such as those found in blood), or in certain organelles such as the endoplasmic reticulum and mitochondrial intermembrane space where disulfides can be introduced.

Sulfur redox chemistry is very important biologically. As described above, the sulfur in cysteine is redox-active. Hence, it can exist in various states, depending on the local redox environment and the presence of oxidizing and reducing agents. A potent oxidizing agent that can be made in cells is hydrogen peroxide, which can lead to more drastic and irreversible chemical modifications to the Cys side chains. Suppose a reactive Cys is important to protein function. In that case, the function of the protein can be modulated (sometimes reversibly, sometimes irreversibly) with various oxidizing agents, as shown in Figure 3.1.21.

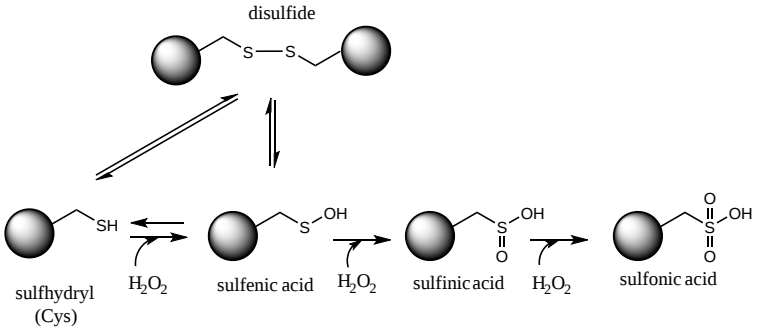


Figure 3.1.20: Reaction of Cysteine with H_2O_2

3.1.15: Reactions of Histidine

Histidine is one of the most important bases at physiological pH. Remember from introductory chemistry that for any acid/conjugate base pair, the pKa and pKb of the acid and base are related by this expression:

$$pKa + pKb = 14$$

Table 3.1.3 below shows the pKa and pKb of three amino acids.

amino acid	pKa	pKb
Histidine	6.5	7.5
Lysine	10.5	3.5
Arginine	12.5	1.5

Table 3.1.3: pKa and pKb values for three amino acid side chains

The deprotonated forms of Lys and Arg with lower pKbs are much stronger bases than the deprotonated form of His, so at physiological pH, they would always be protonated (unless their local environment lowers their pKa and pKb values). In contrast, His exists in both protonated and deprotonated states at physiological pH, so it can readily gain a proton and act as a general base in reactions.

His can exist as two tautomers, as shown in Figure 3.1.22. NMR studies show that in model peptides, the proton predominantly is on the ε_2, N_3, or tele N in the imidazole ring, as it has a pK_a 0.6 units higher than δ_1, N_1, or pro N.

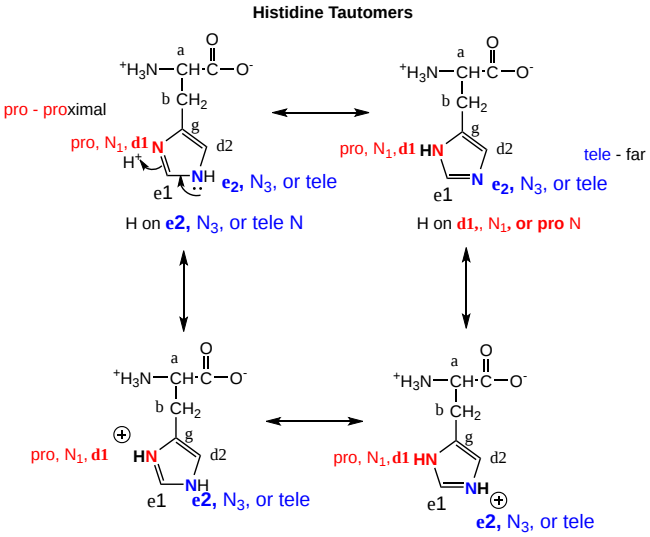


Figure 3.1.22: Histidine tautomers

The nitrogen atom in a secondary amine might be expected to be a stronger nucleophile than a primary amine through electron release to that N in a secondary amine. Opposing this effect is the steric hindrance by the two attached Cs of the N on attach on an electrophile. However, in His, this steric effect is minimized since the 2Cs are restrained by the ring. With a pKa of about 6.5, this amino acid is one of the strongest available bases at physiological pH (7.0). Hence, it can often cross-react with many reagents used to modify Lys side chains. His reacts with reasonably high selectivity with diethylpyrocarbonate as shown in Figure 3.1.23.

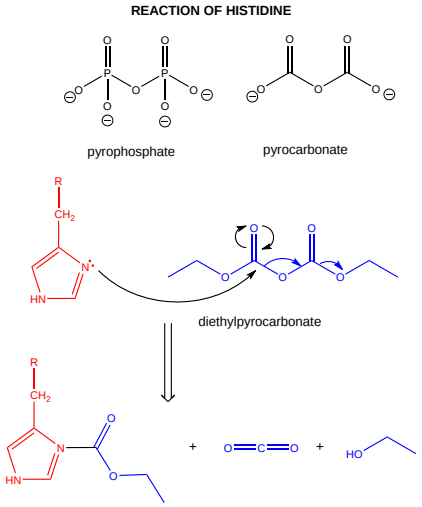


Figure 3.1.23: Reaction of histidine with diethylpyrocarbonate

3.1.16: In vivo Post-Translational Modification of Amino Acids

Amino acids in naturally occurring proteins are also subjected to chemical modifications within cells. These modifications alter the properties of the amino acid, which can alter the structure and function of the protein. Most chemical modifications made to proteins within cells occur after the protein is synthesized in a process called translation. The resulting chemical changes are termed post-translational modifications. Several are shown in Figure 3.1.24. Simple acid/base reactions are included, but these are not considered examples of post-translational modifications.

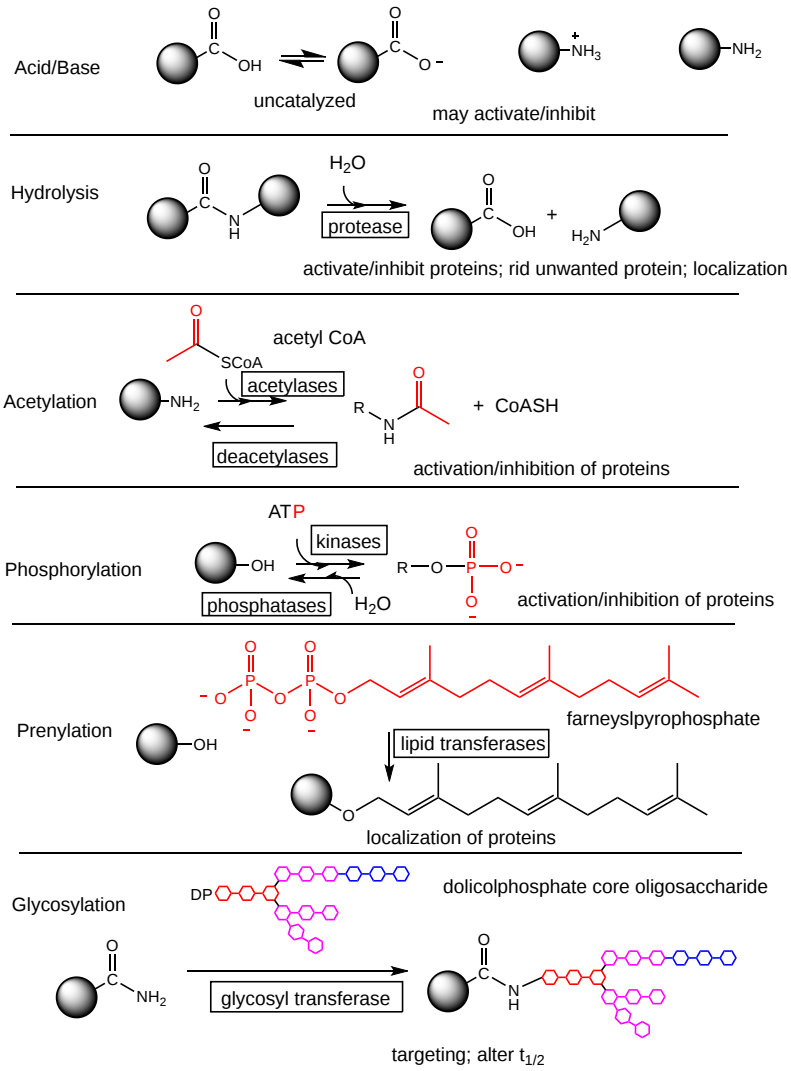


Figure 3.1.24: Common post-translational modifications of protein

There are 100s of PTMs, and many are part of an elaborate system within a cell to respond to both external (hormones, neurotransmitters, nutrients, metabolites) and internal chemical signals. The PTMs (like phosphorylation, acetylation, etc.) and their removal by enzymes are part of an elaborate cell signaling system that we will explore in great detail in Chapter 28. However, not all PTMs are benign. Examples include glycation, oxidation, citrullination, and carbonylation of protein side chains. These are often increased during periods of inflammatory stress (both acute and chronic). These modified proteins are degraded within the cell to short peptides that retain the chemical modification. Unfortunately, these can be recognized by the immune system as foreign, which leads to an immune response against self and autoimmune disease. One potentially deleterious PTM is the carboxyethylation of cysteine, catalyzed by the enzyme cystathionine β-synthase as shown in Figure 3.1.25 below.

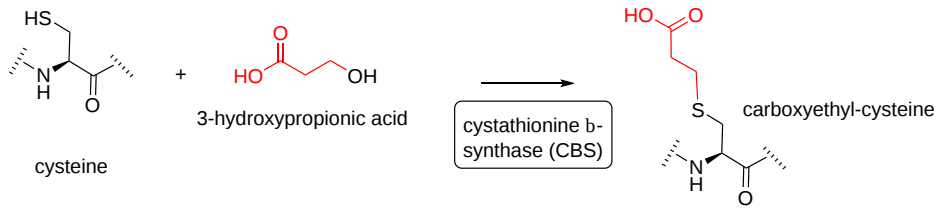


Figure 3.1.25: Carboxyethylation of cysteine

The product is very similar to the carboxymethylation of cysteine shown in Figure 13 above. The modifying reagent, 3-hydroxypropionic acids, is a metabolite released by microbes found in the gut. This modification has been shown to produce an autoimmune response in the disease ankylosing spondylitis.

3.1.17: Summary

This chapter provides an extensive overview of proteins, their building blocks, and the chemical principles that govern their structure and function. It begins by emphasizing that proteins are not only among the most abundant macromolecules in living systems, but also the most functionally diverse. Proteins serve many roles—including structural support, catalysis, regulation, transport, and defense—and their diversity arises from the linear polymerization of alpha-amino acids linked via peptide bonds.

Key topics include:

- **Alpha-Amino Acid Structure and Protein Primary Structure:**
  Every protein is composed of 20 naturally occurring alpha-amino acids, each sharing a common backbone (an α-amino group, an α-carboxyl group, and a hydrogen atom) and a unique R-group that defines its chemical properties. The chapter clarifies the process of peptide bond formation (and hydrolysis) through nucleophilic attack, and it dispels common misconceptions about the free nature of the terminal groups in a protein chain.
- **Amino Acid Side Chain Properties:**
  The diverse chemical nature of proteins is largely due to the variability of the amino acid side chains, which can be classified as nonpolar (hydrophobic), polar uncharged, acidic, or basic. These classifications are critical for understanding protein folding, stability, and function. The chapter introduces hydropathy scales, such as the Kyte-Doolittle and Hopp-Woods scales, to quantitatively assess the relative hydrophobicity or hydrophilicity of side chains, providing insight into protein topology.
- **Stereochemistry and Chirality:**
  Except for glycine, all amino acids are chiral and naturally occur as L isomers in proteins. The chapter contrasts the D/L nomenclature with the R/S system and explains why biochemists favor the former, particularly when describing the stereochemistry of amino acids in the context of protein structure.
- **Acid-Base Chemistry and Ionization:**
  The ionizable groups on amino acids, including the α-amino, α-carboxyl, and various side chains, determine the overall charge and reactivity of proteins. The Henderson–Hasselbalch equation is introduced as a tool to predict the protonation state of these groups under varying pH conditions, leading to the concept of the isoelectric point (pI) — the pH at which a protein has no net charge.
- **Chemical Reactivity of Side Chains:**
  Beyond structural roles, the chemical reactivity of amino acid side chains is central to protein function. The chapter details how key side chains—such as those of lysine, cysteine, and histidine—participate in nucleophilic reactions, form covalent modifications (e.g., Schiff bases), and are targeted by specific chemical reagents. This reactivity is foundational for both enzyme catalysis and experimental methods used to probe protein structure and function.
- **Post-Translational Modifications (PTMs):**
  The chapter concludes by discussing PTMs, chemical modifications that occur after protein synthesis. These modifications, including phosphorylation, acetylation, glycosylation, and oxidation, can dramatically alter a protein's activity, stability, and interactions. While many PTMs are critical for normal cellular signaling, aberrant modifications may contribute to disease.

Overall, the chapter integrates structural biology with chemical reactivity, providing a comprehensive framework that links the primary structure of proteins to their three-dimensional architecture and dynamic functions in the cell. This foundation is essential for understanding advanced topics in enzymology, protein engineering, and molecular regulation.

3.2: The Structure of Proteins- An Overview

Learning Goals (ChatGPT o1, 1/25/25)

- **Understand the Mechanism of Peptide Bond Formation:**
  - Explain the process of dehydration synthesis that links amino acids, including the role of water loss in forming the amide (peptide) bond.
  - Describe the chemical activation of the carboxylic acid group (via ATP and formation of a high-energy intermediate) that facilitates nucleophilic attack by the amino group.
  - Recognize common misconceptions related to protonation states in peptide bond formation and clarify the proper chemical representation.
- **Comprehend the Role of the Ribosome and Genetic Information:**
  - Describe how the ribosome, as a ribonucleoprotein nanoparticle, catalyzes peptide bond formation during translation.
  - Connect the sequence information encoded in mRNA (and ultimately genomic DNA) to the primary sequence of a protein.
  - Understand the directionality of protein synthesis (N-terminus to C-terminus) and its implications for protein structure.
- **Define Protein Primary Structure:**
  - Identify the primary structure of a protein as its unique linear sequence of amino acids.
  - Explain how the order of amino acids determines the eventual folding and function of the protein.
  - Differentiate between peptides and proteins based on size, synthesis, and structural criteria.
- **Explore the Diversity of Protein Sequences and Structures:**
  - Quantify the vast number of potential protein sequences even for short peptides and appreciate the diversity generated by 20 different amino acids.
  - Discuss the hierarchical organization of protein structure (primary, secondary, tertiary, and quaternary) and the role of primary structure as the foundation for higher-order folding.
  - Use examples (e.g., hydroxynitrile lyase) to illustrate how distinct regions of the primary sequence can adopt specific secondary structures (α-helices, β-sheets, coils) and contribute to overall tertiary and quaternary arrangements.
- **Connect Chemical Structure to Protein Function:**
  - Analyze how the primary sequence and covalent peptide bonds establish the framework for protein folding and function.
  - Discuss how post-translational modifications and disulfide bond formation (both intra- and inter-chain) further stabilize protein structure and mediate functional interactions.
  - Relate the principles of peptide bond formation and sequence variability to the extraordinary range of protein functions in biological systems.

These learning goals will guide students in integrating chemical, structural, and genetic principles that underlie peptide synthesis and protein structure, setting the stage for deeper exploration of protein folding, function, and regulation in later chapters.

3.2.1: Peptide Bond Formation and Primary Protein Structure

Proteins are polymers of amino acids that fold into shapes that confer function on the proteins. In biological systems, the amino acids are linked by a large ribonucleic acid/protein nanoparticle called the ***ribosome***. Thus, as the amino acids are linked together to form a specific protein, they are placed within a specific order dictated by the genetic information contained within a specific type of RNA called messenger RNA (mRNA). The mRNA sequences are encoded in the genomic DNA sequence. The specific ordering of amino acids is the protein's ***primary sequence***. The translation mechanism used by the ribosome to synthesize proteins will be discussed in detail in Chapter 26.

The amino acids are linked together using ***dehydration synthesis*** (loss of water) reaction that connects the carboxylic acid of the upstream amino acid with the amine functional group of the downstream amino acid to form an amide linkage (Figure 2.10). You

will remember from other chemistry courses that forming an amide from a carboxylic acid (thermodynamically stable) and an amine requires activating the carboxylic acid end to form a derivative with a better-leaving group. This carbonyl of the modified end serves as an electrophile in the attack of the amine nitrogen, a nucleophile, in a nucleophilic substitution reaction. The activation reaction, which we will discuss in subsequent chapters, involves the transfer of a phosphate from a phosphoanhydride, ATP, to the carboxylic acid group to form a mixed anhydride with the phosphate serving as a leaving group. Note that the reverse reaction is ***hydrolysis*** and requires the incorporation of a water molecule to separate two amino acids and break the amide bond. Notably, the ***ribosome*** serves as the enzyme that mediates the dehydration synthesis reactions required to build protein molecules, whereas a class of enzymes called ***proteases*** is required for protein hydrolysis.

A **peptide bond** is the amide linkage between amino acids in a protein. Subsequent amino acids will be added to the carboxylic acid terminal of the growing structure. Proteins are always synthesized directionally, starting with the amine and ending with the carboxylic acid tail. New amino acids are always added onto the carboxylic acid tail, never onto the amine of the first amino acid in the chain. The ribosome dictates the directionality of protein synthesis. Figure 3.2.1 below shows an overly simplistic version of the reaction that produces the amide bond.

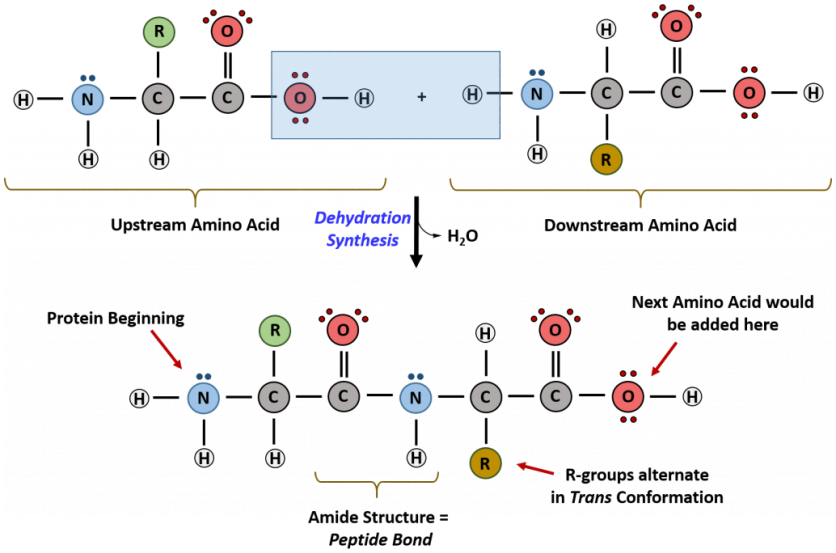


Figure 3.2.1: Formation of the Peptide Bond. The addition of two amino acids to form a peptide requires dehydration synthesis. (Copyright; author via source)

Please note two features of the reaction as shown in the diagram:

1. The activation step (phosphorylation of the carboxylic acid end of the amino acid by ATP) is not shown.
2. The reaction is shown with an unlikely protonation state. If the carboxyl group is protonated, which would occur at a low pH, the amine would also be protonated and should correctly be shown as RNH_3^+. The protonation state in the above figure was chosen to emphasize the loss of H_2O (dehydration) in the reaction. Many textbooks that aren't rigorously based in chemistry show unlikely protonation states for this reaction. By discussing this now, we hope to highlight common mistakes and misconceptions found in many resources.

Figure 3.2.2 shows a generic structure from a longer peptide or protein.

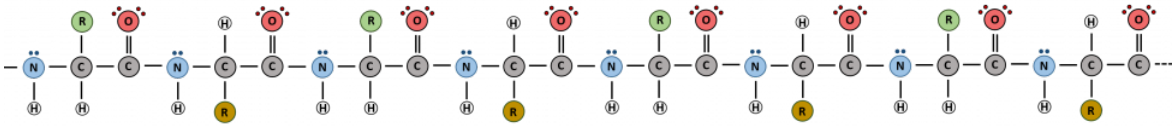


Figure 3.2.2: Peptides and Proteins are macromolecules built from long chains of amino acids joined together through amide linkages. The order and nature of amino acids in the primary sequence of a protein determine the folding pattern of the protein based on the surrounding environment of the protein

Proteins range in size from around 50 amino acids in length to rare proteins with molecular weights in the millions. One example is the human protein titin (also called connectin), a muscle protein. The human version has over 34,000 amino acids and a molecular weight of over 3.9 million! A polyketide synthase (PKS or a PKZILLAs) has 45,212 amino acids with a molecular

weight of 4.7 million. "Giant" proteins, up to 85,804 amino acids, are encoded by some bacteria in the phylum Omnitrophota found in wastewater and hot pools. However, only pieces of the protein are found.

Some consider structures with fewer than 50 amino acids as **peptides** (Figure 2.13). Others suggest that 40-50 amino acid structures should be considered **small proteins**. One way to differentiate them is by how they are synthesized in vivo. Storz et al consider polypeptides to be small proteins if they are encoded in the genome by a continuous stretch of DNA base in an "open reading frame" (doi: 10.1146/annurev-biochem-070611-102400). Peptides, on the other hand, could be structures that are:

- "intrinsically disordered" with no definite fold,
- derived from proteins by proteolysis and/or
- not synthesized by ribosomes

As genomes were sequences and annotated, some arbitrary parameters were set. For the yeast genome, annotated proteins were defined as those made from an open reading frame (ORF) in the DNA sequence that encodes the protein, leading to a polypeptide of 100 amino acids (which, on average, has a molecular weight of 11,000). If no cutoff were used, the number of proteins encoded by the genome would be huge. Submissions of DNA sequences to the NIH GenBank must encode proteins no smaller than about 66 amino acids (MW about 7250). Even this ignores small proteins that have been isolated and characterized from cells. So the cutoff of 50 amino acids (MW about 5500) derived from open reading frames seems like the best arbitrary cutoff in the transition from peptides to proteins.

The definition of a protein as encoded in an "open reading frame" in the DNA/RNA is contingent on how you define an open reading frame. Evidence suggests that many proteins are made from atypical or noncanonical open reading frames (ncORFs). This study found that at least 25% of 7,264 ncORFs were transcribed and translated into over 3,000 peptides/proteins. Most DNA/RNA sequences were less than 100 codons (or the equivalent number of amino acids). Most were also within long noncoding RNAs (see RNA Chapter) or untranslated regions of messenger RNAs. Their functional significance is, in most cases, uncertain, yet their presence would expand the number of protein-encoding genes in the human genome. These sequences are available in two databases: GENCODE, which can identify and classify gene features in human and mouse genomes based on biological evidence, and PeptideAtlas, which houses peptide structures determined by mass spectroscopy. It's difficult to differentiate peptides transcribed from ncORFs from the 1000s of peptides produced in cells from proteolysis and other means. The new sequences, found in what has been called the "dark proteome," add 1000s of protein to the previous total of around 20,000 human protein-coding genes.

Due to the large pool of amino acids that can be incorporated at each position within the protein, billions of different possible protein combinations can be used to create novel protein structures! For example, think about a tripeptide made from this amino acid pool. At each position, 20 different options can be incorporated. Thus, the total number of resulting tripeptides possible would be 20 X 20 X 20 or 20^3, or 8,000 different tripeptide sequences! Now, think about how many options there would be for a small peptide containing 40 amino acids. There would be 20^{40} options or a mind-boggling 1.09 X 10^{52} potential sequence options! Each of these options would vary in the overall protein shape, as the nature of the amino acid side chains helps to determine the interaction of the protein with the other residues in the protein itself and with its surrounding environment.

Nearly 200,000 3D structures of biomacromolecules are known, and over a million have been determined using artificial intelligence computer programs. How can we simplify our understanding of the diversity of protein structures? Is each structure unique? What do they have in common?

To simplify and inform our understanding of the diversity of biological organisms, we place them into groups (from domains and kingdoms to genuses and species), based on common characteristics. Likewise, proteins are divided into a hierarchy of structures with increasing information content. This overview describes the four basic levels of protein structure: primary (10), secondary (20), tertiary (30), and quaternary (40). Each will be probed in greater detail in the next chapter. These classes of structures will be illustrated below with a protein named **hydroxynitrile lyase** (5Y02). (This protein has been simplified to illustrate key structural features, which will be described at the end.)

3.2.2: Primary (1^0) Structure: the amino acid sequence of a protein.

A protein's primary (10) structure is simply the amino acid sequence of a protein written from N- to C-terminal. It does not require visualization to describe it. Consider two different short continuous sequences from the hydroxynitrile lyase:

- Gln-Lys-Gln-Ile-Asp-Gln-Ile or in single letter code QKQIDQI. This is the sequence for amino acids 20-26 in the protein. This stretch of 1^0 structure has multiple repeated amino acids.

- Asp-Leu-Gly-Pro-Ala-Val or in single letter code DLGPAV. This is the sequence for amino acids 48-53 in the protein. This stretch of 1^0 structure does not contain repetitive amino acids.

A 2-D line drawing of the sequence offers more information but does not provide information about the conformation of these sections of 1^0 structures within a given protein. These can be shown in Figure 3.2.3, in which the protein's overall structure is shown in grey sticks with short stretches of primary structure shown in colored spacefill and 2D line drawings.

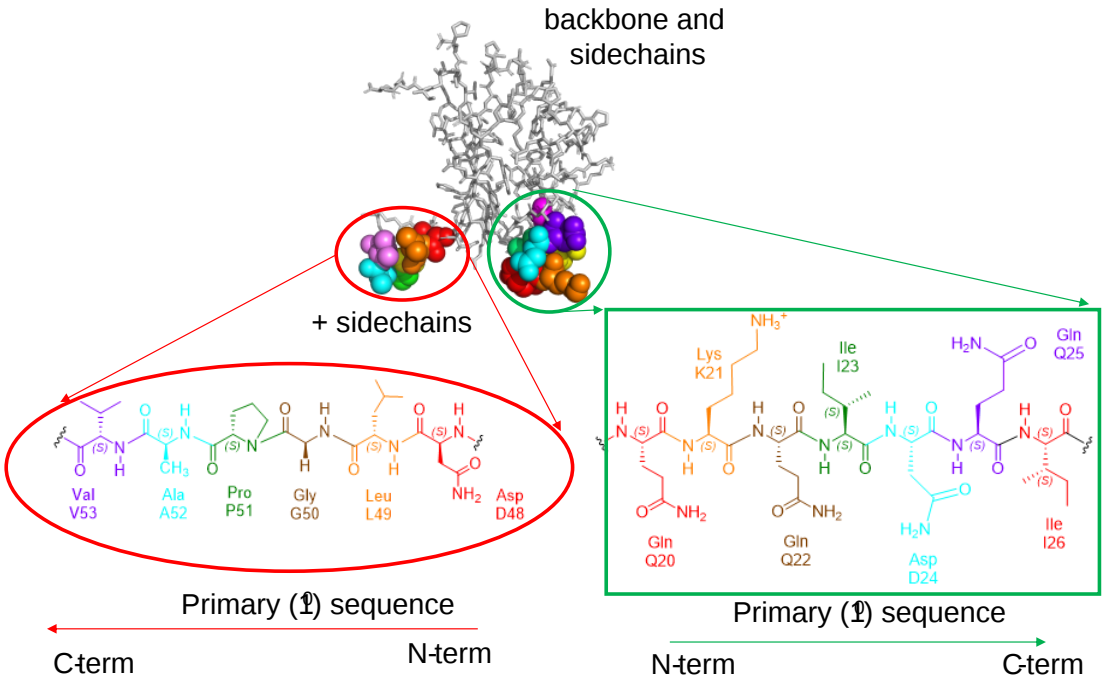


Figure 3.2.3: Alternative renderings of a "primary" sequence within a protein

3.2.3: Secondary (2^0) and Tertiary (3^0) Structures

Secondary (2^0) structures are repetitive structures within a protein held together by hydrogen bonds between amide Hs and carbonyl Os in the backbone main chain atoms. It's most easily examined through the specific rendering of the overall **tertiary (3^0)** or 3-D protein structure. Five different renderings showing the 3D (the **tertiary**) structure of the protein are shown in Figure 3.2.4.

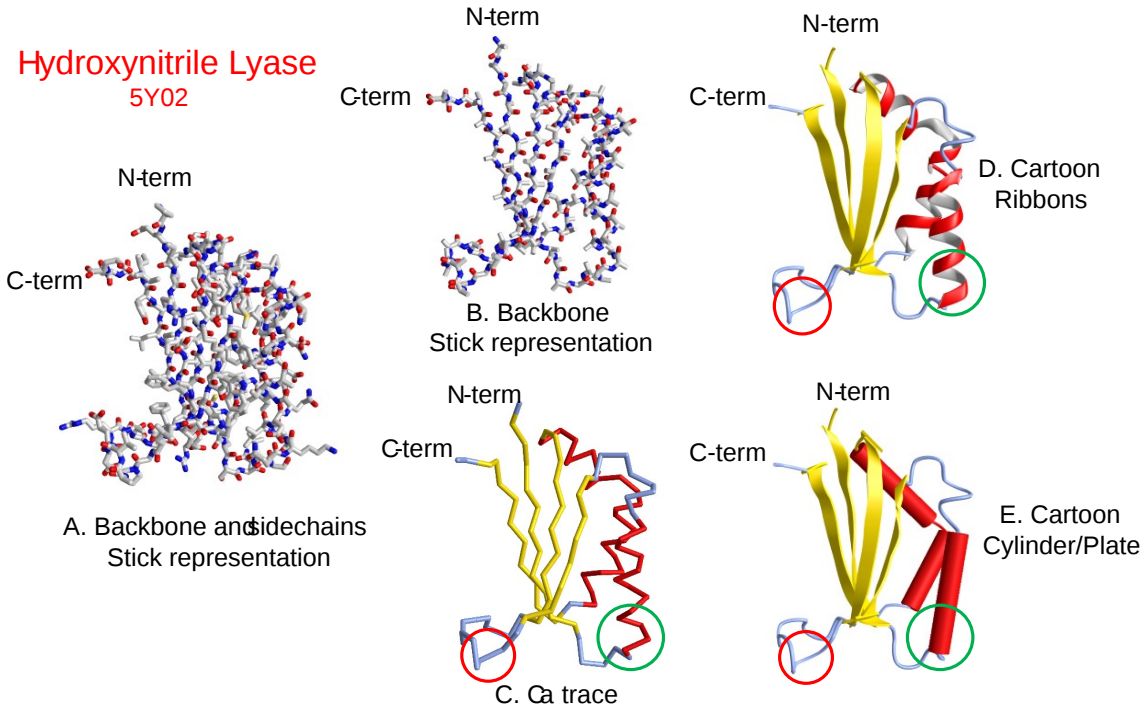


Figure 3.2.4: Alternative renderings of a protein

Representation **A** shows a stick drawing of the protein with red indicating bonds to oxygen and blue bonds to nitrogen. No covalent bonds to hydrogen are shown as hydrogen atoms are too small to be detected using common techniques to determine the structures of such large molecules. It looks like a complicated mess of bonds, so understanding unique features with the 30 structure of the protein is difficult. Representation **B** shows just the backbone of the protein. The outline of how the protein twists and turns in space becomes more evident. The N- and C-terminal ends are more clearly seen.

Representation **C** shows just the bonds connecting the alpha C atoms of each amino acid. The protein's overall topology is now clearly evident. If you follow the chain from the N- to C-terminal ends, it should be evident that there are regularities in the conformations of the protein chain. The individual yellow zig-zags are called **beta** strands. These strands appear elongated and aligned with other beta strands to form a larger **beta sheet**. The sheet is held together through hydrogen bonds between backbone amide Hs and carbonyl Os on adjacent strands. Beta strands are a type of **secondary structure**.

The red zig-zag lines represent another type of secondary structure called the **alpha helix**. Hydrogen bonds hold the helix together between amide Hs and carbonyl Os within a single continuous strand. The backbone of the alpha helix appears less elongated than in a beta-strand as it is wound into a coil (the alpha helix) along a central axis. If you took tweezers (using atomic force microscopy) and pulled on the helix, it could stretch and become more elongated like the beta strands.

The remaining protein alpha carbon chain shown in blue is less regular. However, it is still ordered as it propagates through space in a **random coil**. It mainly adopts a fixed conformation but has more conformational flexibility than alpha helices and beta sheets. The alpha helices and beta strands (sheets) are examples of secondary structures.

Representations **D** and **E** are cartoon drawings clearly showing alpha helices (red), beta stands, and sheets (yellow). It would be extremely difficult to discern alpha or beta secondary structures with stick representations showing all the bonds in a protein. Some atoms must be removed visually (not literally) to see the protein backbone's repetitive propagation through the overall structure. A cartoon view alone would not be useful if you aimed to understand the disposition of side chains in a small part of a protein. Modeling programs allow mixed rending of a protein to include cartoon and stick representations.

Secondary structures, held together by hydrogen bonds between backbone atoms, are characterized by repetitive changes in the chain propagation angle between connected amino acids in an alpha helix and a beta-strand. In a given beta-strand, the relative change in the propagation angle is nearly 0^0 compared to a much larger angular change required to bend the amino acid backbone into an alpha helix. Here is the IUPAC definition of secondary structure. We added the word "repetitive" to show that random coils are not an example of secondary structures.

Definition: Secondary Structure (from the IUPAC Gold Book)

The [repetitive] conformational arrangement (α-helix, β-pleated sheet, etc.) of the backbone segments of a macromolecule, such as a polypeptide chain of a protein without regard to the conformation of the side chains or the relationship to other segments.

3.2.4: Quaternary Structure

Separate protein chains often interact through noncovalent interactions and sometimes through disulfide bond formation between free cysteine side chains on different chains to form dimers, trimers, tetramers, octamers, etc. Dimers can be homodimers (if the two chains are identical) or heterodimers (if they are different). The example in the section, hydroxynitrile lyase, forms a homodimer, as shown in Figure 3.2.5. The left image shows a cartoon version, with one monomer in orange and the other identical monomer in green. The right image shows a translucent surface representation of the dimer, with the cartoon image underneath.

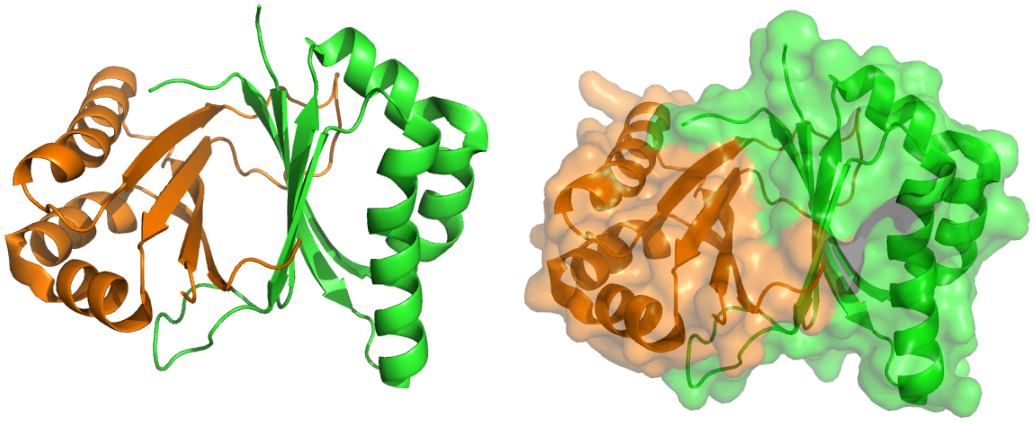


Figure 3.2.5: Dimer of hydroxynitrile lyase

The mixed-rendered image on the right shows a translucent surface image of each monomer, and underneath the cartoon image

- primary structure: the linear amino acid sequence of a protein
- secondary structure: regular repeating structures arising when hydrogen bonds between the peptide backbone amide hydrogens and carbonyl oxygens occur at regular intervals within a given linear sequence (strand) of a protein or between two adjacent strands

Disulfide bonds within individual chains and between them stabilize both tertiary and quaternary structures of both peptides and proteins. These are illustrated in Figure 3.2.6.

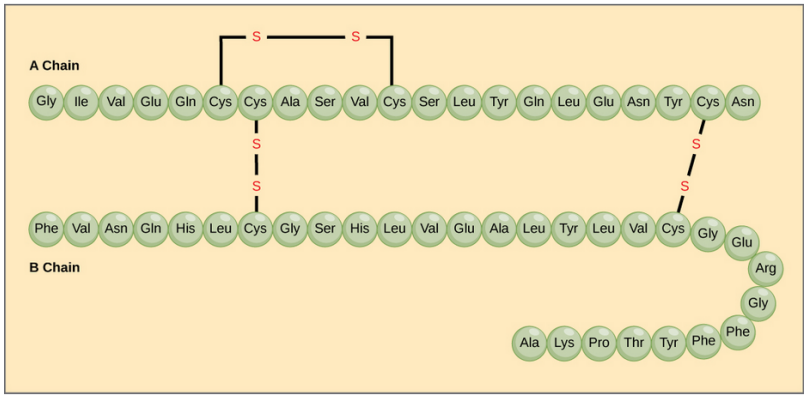


Figure 3.2.6: **Disulfide Bonds.** Disulfide bonds are formed between two cysteine residues within a peptide or protein sequence or between different peptide or protein chains. The two peptide chains that form the hormone insulin are depicted in the example above. Disulfide bridges between the two chains are required for the proper function of this hormone to regulate blood glucose levels. Image by: CNX OpenStax via Wikimedia Commons

3.2.5: Summary

Chapter Summary

This chapter delves into the foundation of protein structure by examining peptide bond formation and the concept of primary structure. It begins by outlining how proteins, as polymers of amino acids, are synthesized by the ribosome. This complex ribonucleoprotein machine translates the genetic code carried by mRNA into a specific linear sequence of amino acids. This sequence, known as the protein's primary structure, is determined by the genomic DNA and dictates the protein's ultimate three-dimensional structure and function.

Key topics include:

- **Peptide Bond Formation:**
  The chapter explains that amino acids are linked together via dehydration synthesis—a process that forms an amide (peptide) bond by losing water. It discusses the necessity of activating the carboxylic acid group (typically through phosphorylation using ATP) to create a better leaving group, thereby allowing the nucleophilic attack by the amine group of the incoming amino acid. The role of the ribosome in catalyzing this reaction and common misconceptions regarding protonation states during the reaction is clarified.
- **Primary Protein Structure:**
  Emphasis is placed on the importance of the linear sequence of amino acids. The chapter details how the unique ordering of amino acids in a protein—ranging from short peptides to giant proteins like titin—forms the basis for its folding into higher-order structures (secondary, tertiary, and quaternary). This primary structure not only defines the chemical properties of the protein but also underlies its functional diversity in biological systems.
- **Diversity and Definition of Proteins vs. Peptides:**
  The discussion highlights the enormous diversity possible from 20 amino acids and how even a short peptide can have millions of potential sequences. It also addresses the criteria used to distinguish between peptides and proteins, including size and the synthesis mechanism (continuous open reading frames versus proteolytic products).
- **Hierarchy of Protein Structure:**
  Finally, the chapter introduces the concept of hierarchical protein organization—starting with the primary structure, moving through regular secondary structures (such as α-helices and β-sheets stabilized by hydrogen bonds), and culminating in complex tertiary and quaternary structures that often involve additional stabilizing elements like disulfide bonds.

Overall, this chapter lays the groundwork for understanding how the precise order of amino acids is essential for protein folding and function, setting the stage for more detailed studies of protein structure and dynamics in subsequent chapters.

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3.3: Protein Purification

Learning Goals (ChatGPT o1, 1/25/25)

- **Explain the Rationale for Protein Purification:**
  - Describe why isolating a single protein from complex cellular mixtures is essential for structural and functional studies.
- **Differentiate Between Preparative and Analytical Purification:**
  - Distinguish the objectives, techniques, and quality control measures used in preparative versus analytical purification.
- **Outline Sample Processing Techniques:**
  - Identify various cell lysis methods (e.g., freeze/thaw, sonication, detergent treatment) and discuss how centrifugation separates soluble proteins from cellular debris.
- **Understand Precipitation and Dialysis Methods:**
  - Explain the role of ammonium sulfate precipitation and dialysis in concentrating proteins and removing contaminants.
- **Apply Chromatography Principles:**
  - Compare and contrast different chromatographic techniques (size exclusion, ion exchange, hydrophobic interaction, and affinity chromatography) based on protein properties such as size, charge, hydrophobicity, and binding affinity.
- **Interpret Chromatographic Data:**
  - Analyze elution profiles, calculate distribution coefficients, and evaluate parameters like void volume and stationary phase volume.
- **Quantitatively Evaluate Purification Efficiency:**
  - Calculate and interpret metrics such as total protein, total activity, specific activity, yield, and fold-purification to assess the success of a purification scheme.
- **Describe the Theory of Electrophoresis:**
  - Explain how charged particles move under an electric field, and relate factors like electrophoretic mobility to charge, size, and shape (including concepts like the Stokes radius).
- **Distinguish Native and Denaturing Electrophoresis:**
  - Compare techniques such as native PAGE and SDS-PAGE, and understand how SDS alters protein charge and conformation to facilitate molecular weight-based separation.
- **Integrate Isoelectric Focusing and Two-Dimensional Electrophoresis:**
  - Understand how pH gradients in isoelectric focusing separate proteins by their isoelectric points and how 2-DE combines IEF with SDS-PAGE for high-resolution analysis.
- **Interpret Gel Staining and Detection Methods:**
  - Evaluate different protein detection methods (Coomassie Blue, silver staining, autoradiography, Western blotting) and their applications in visualizing protein bands.
- **Relate Biochemical Principles to Instrumentation:**
  - Explain how HPLC, FPLC, and mass spectrometry (e.g., MALDI-TOF MS) integrate with protein purification protocols to provide structural and functional insights.
- **Understand the Role of Protein Tagging and Peptide Synthesis:**
  - Describe how molecular tags (e.g., His, FLAG, GFP) and solid-phase peptide synthesis facilitate purification and identification of proteins.
- **Critically Analyze Experimental Design:**

- Identify potential sources of error in purification protocols (e.g., proteolysis, non-specific binding) and propose strategies to optimize yield and purity.

- **Apply Theoretical Concepts to Practical Scenarios:**
  - Use mathematical relationships (such as $U = Q/(6\pi\eta Rs)$) to predict and analyze protein migration behavior in electrophoretic systems.

These goals aim to provide a comprehensive understanding that links the theory and practice of protein purification and electrophoresis, preparing students for advanced research and practical applications in biochemistry.

3.3.1: Introduction

A protein or other biological macromolecule must be purified before rigorously being studied structurally and functionally. Purifying a single protein from a mixture of as many as 10,000 other cellular or tissue proteins, each containing the same 20 naturally occurring amino acids linked in different orders, is difficult. Proteins differ in size (how many amino acids), charge (how many positively and negatively charged amino acids), sequence, and presence of specific binding sites on the proteins. Any technique that could be used to purify protein must be based on these inherent differences. Once the protein is purified, it must be analyzed by a spectral or electrophoretic technique.

Protein purification is a series of processes intended to isolate and purify a single protein or complex from cells, tissues, or whole organisms. Protein purification is vital for characterizing the function, structure, and interactions of the protein of interest. Separation steps usually exploit differences in protein size, physical-chemical properties, binding affinity, and biological activity.

Protein purification is either *preparative* or *analytical*. **Preparative purifications** aim to produce a relatively large quantity of purified proteins for subsequent use. Examples include the preparation of commercial products such as enzymes (e.g., lactase), nutritional proteins (e.g., soy protein isolate), and certain biopharmaceuticals (e.g., insulin). Many steps and quality control are required to remove other host proteins and biomolecules that could threaten the patient's health. **Analytical purification** produces a relatively small amount of a protein for various research or analytical purposes, including identification, structural characterization, and studies of the protein's structure, post-translational modifications, and function.

The choice of a starting material is key to the design of a purification process. In plants or animals, a particular protein usually isn't distributed homogeneously throughout the organism; different organs or tissues have higher or lower protein concentrations. Using tissues or organs with the highest concentration decreases the volumes needed to produce a given amount of purified protein. A protein of low abundance or high commercial value is often made using recombinant DNA technology. These techniques will be discussed in greater detail in Chapter 5.

3.3.2: Sample Processing

If the organism does not secrete the protein of interest into the surrounding solution, the first step of each purification process disrupts the cells containing the protein. Depending on how fragile the protein is, one of several techniques could be used, including repeated freezing and thawing, sonication, homogenization by high pressure (French press), homogenization by grinding (bead mill), and permeabilization by detergents (e.g., Triton X-100) and/or enzymes (e.g., lysozyme). Finally, the cell debris can be removed by centrifugation so that the proteins and other soluble compounds remain in the supernatant.

Proteases released during cell lysis cleave the proteins in the solution. As the protein of interest may be sensitive to proteolysis, it is important to proceed quickly and conduct many steps at low temperatures to reduce unwanted proteolysis. Alternatively, one or more protease inhibitors can be added to the lysis buffer immediately before cell disruption. Sometimes, it is also necessary to add DNase to reduce the viscosity of the cell lysate caused by a high DNA content.

3.3.3: Centrifugation

Centrifugation is a process that uses centrifugal force to separate mixtures of particles of varying masses or densities suspended in a liquid. When a vessel (typically a tube or bottle) containing a mixture of proteins or other particulate matter, such as bacterial cells, is rotated at high speeds, the inertia of each particle yields a force in the direction of the particle's velocity proportional to its mass. The tendency of a given particle to move through the liquid because of this force is offset by the resistance the fluid exerts on the particle. The net effect of "spinning" the sample in a centrifuge is that massive, small, and dense particles move outward faster than less massive particles or particles with more "drag" in the liquid. When suspensions of particles are "spun" in a centrifuge, a "pellet" may form at the bottom of the vessel enriched for the most massive particles with low drag in the liquid.

Non-compacted particles remain mostly in the liquid "supernatant" and can be removed from the vessel, separating the supernatant from the pellet. The angular acceleration applied to the sample determines the centrifugation rate, typically measured compared to *g*, the gravitational constant. If samples are centrifuged long enough, the vessels' particles will reach equilibrium, wherein the particles accumulate specifically at a point in the vessel where their buoyant density is balanced with centrifugal force. Such an "equilibrium" centrifugation can allow extensive particle purification.

In ***sucrose gradient centrifugation***, a linear concentration gradient of sugar (typically sucrose, glycerol, or a silica-based density gradient media, like Percoll) is generated in a tube such that the highest concentration is on the bottom and the lowest on top. Percoll is a trademark owned by GE Healthcare companies. A protein sample is then layered on the gradient and spun at high speeds in an ultracentrifuge. This causes heavy macromolecules to migrate toward the bottom of the tube faster than lighter material. During centrifugation, in the absence of sucrose, as particles move farther and farther from the center of rotation, they experience greater centrifugal forces (the further they move, the faster they move). However, the useful separation range within the vessel is restricted to a small observable window. A properly designed sucrose gradient will counteract the increasing centrifugal force so the particles move proportionately to their time in the centrifugal field. After separating the protein/particles, the gradient is fractionated and collected. These are described in Figure 3.3.1.

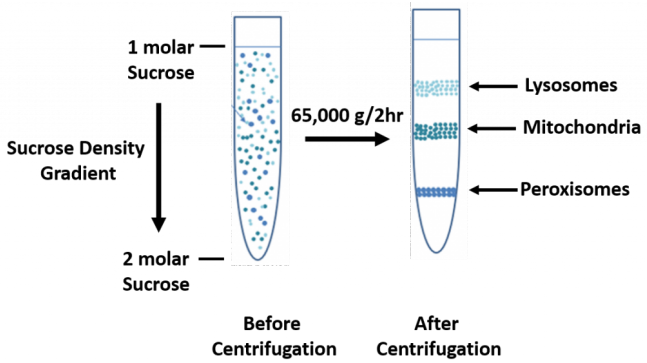


Figure 3.3.1: Sucrose Density Gradient. *Image derived from Michel Awkal*

3.3.4: Precipitation and Differential Solubilization

In bulk protein purification, precipitation is a common first step to isolate proteins using a salt such as ammonium sulfate $(NH_4)_2SO_4$. Ammonium sulfate is often used as it is highly soluble in water, has relative freedom from temperature effects, and typically is not harmful to most proteins. Proteins are precipitated by $(NH_4)_2SO_4$ in their native state, which is important if you need the protein for structure/function studies. Furthermore, dialysis can remove ammonium sulfate as described in Figure 3.3.2.

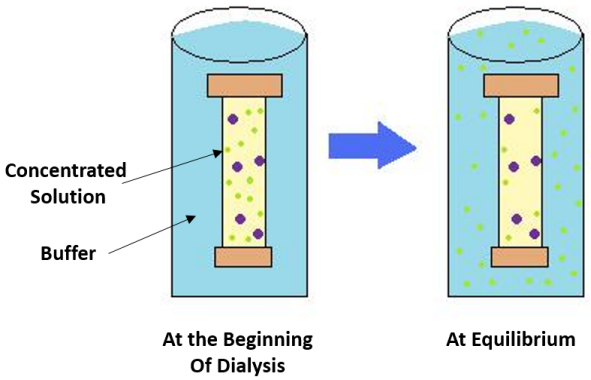


Figure 3.3.2: Dialysis of a macromolecule (blue dots). *Image adapted from Gjk003*

The process of dialysis separates dissolved molecules by their size. The biological sample is placed inside a closed membrane, where the protein of interest is too large to pass through the membrane's pores but through which smaller ions can easily pass. As the solution reaches equilibrium, the ions become evenly distributed throughout the solution while the protein remains concentrated in the membrane. This reduces the overall salt concentration of the suspension.

The mechanism underlying salt precipitation is quite complicated. High concentrations of sodium chloride don't precipitate protein. Other salts like guanidinium chloride unfold proteins and do not lead to precipitation. Salt ions interact with the protein and solvent water in somewhat complicated ways (which we will explore later). We will be satisfied with the empirical observation that ammonium sulfate is the salt of choice to precipitate and concentrate proteins from a solution. One advantage of $(NH_4)_2SO_4$ protein precipitation from solution is that it can be performed inexpensively with very large volumes, so it is used early in many purification proteins. Different proteins precipitate at different $(NH_4)_2SO_4$ concentrations, so differential precipitation is often used. $(NH_4)_2SO_4$ concentrations are increased stepwise until the protein of interest is precipitated.

Some proteins are not soluble in water. These include transmembrane proteins that span cell membranes and large fibrous proteins. Membrane proteins can be solubilized by adding detergents like sodium dodecyl sulfate (SDS), which unfolds the proteins, and octylglucoside or Triton X-100, which keeps the protein structure intact.

3.3.5: Chromatography

Chromatography is used in almost all protein purification methods and is the key to separating a given protein from the 1000s of different proteins in cells and tissues. The separation of proteins on a chromatography column depends on the type of column and the chemical/physical properties of the molecule. There are four main types of chromatographies used to separate proteins:

- **size exclusion** chromatography in which proteins can be separated according to their size/shape or molecular weight
- **ion exchange** chromatography in which proteins are separated by their charge/isoelectric point;
- **hydrophobic interaction** chromatography (similar to reverse phase columns for purifying organic molecules) in which they are separated based on their relative hydrophobicity
- **affinity** chromatography in which proteins are separated based on binding to a ligand covalently attached to a column bead.

The purification protocol generally contains one or more chromatographic steps for preparative protein purification. The basic procedure in chromatography is to flow the solution containing the protein through a column packed with a chromatography resin selected to separate proteins based on a specific property of the protein. Different proteins interact differently with the column material. They can thus be separated by the time required to pass the column or the conditions required to elute the protein from the column. Usually, proteins are detected as they are eluting from the column by measuring the absorbance at 280 nm, at which the aromatic amino acids absorb.

3.3.5.1: Size Exclusion Chromatography (also known as Gel Filtration Chromatography)

This method is used to separate proteins based on size and shape. The chromatography beads have tiny openings/pores into which proteins smaller than the pore diameter can enter. Large proteins that can't enter the pores flow around the beads and elute faster than small ones that enter the pores. They diffuse out of the pores and enter the rest of the moving solvent before getting "trapped" again for a short time in more pores. Eventually, they move through the column and elute at a much greater volume than larger proteins, which can't enter the pores. Thus, proteins will be separated based on size, as illustrated in Figure 3.3.3. The eluate is collected in sequential test tubes (or fractions). The figure below shows the pores as channels that go through the bead. The openings in resin beads should be considered tiny crevices and indentations, not channels.

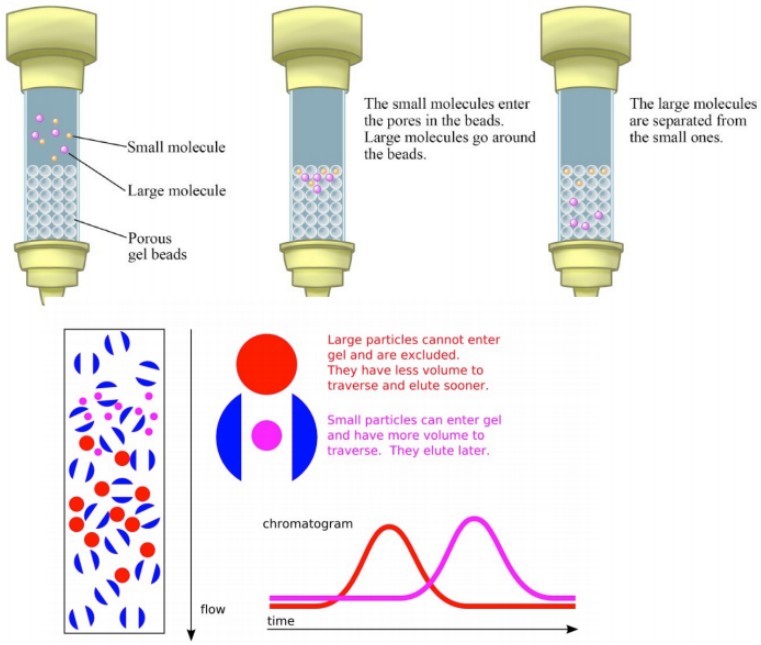


Figure 3.3.3: **Size Exclusion Chromatography.** *Image from Dr. Kevin Ahern and Indira Rajagopal*

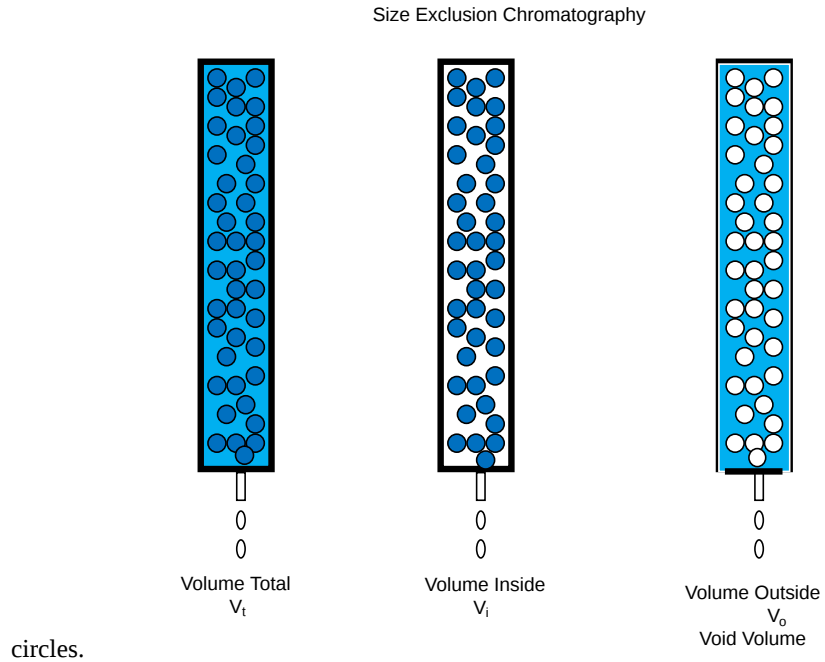
Also known as gel filtration chromatography, it is a low-resolution isolation method involving beads with tiny “pores" that have a precise size distribution. The size is called an “exclusion limit," meaning molecules above a certain molecular weight will not fit into the tunnels. Molecules with sizes larger than the exclusion limit do not enter the tunnels and pass through the column relatively quickly by making their way between the beads. Smaller molecules, which can enter the tunnels, do so and, thus, have a longer path in passing through the column. Because of this, molecules larger than the exclusion limit will leave the column earlier, while smaller molecules that pass through the beads will elute from the column later. This method allows the separation of molecules by their size.

In any chromatography system, there is a mobile and stationary phase. For size exclusion chromatography, the stationary phase is usually a polymerized agarose or acrylamide bead, which contains pores of various sizes filled with the solvent. Let's pretend that the solvent (typically aqueous buffered solution) inside the bead is trapped there and doesn't exchange with the solvent moving around the bead so that it would be part of the stationary phase. The mobile phase is the solvent used to elute the column, which flows around the bead. The chromatography beads are often supplied in dried form, which must be swollen in the solvent before they are packed in the column. The volume of the agarose or acrylamide bead is very small compared to the volume of solution within their hydrated forms.

3.3.5.2: Size and shape effects in size exclusion chromatography

Size-size exclusion chromatography is so common so that we will explore it in greater detail

Several different column volumes can be defined as shown in Figure 3.3.4, where the packed chromatography beads are shown as



circles.

Figure 3.3.4: Define volumes in size exclusion chromatography

If we consider the mass of the beads to offer a *negligible amount to the volume of the bead*, the actual volume in the bead is mostly from the trapped solution, which can be considered the "stationary" phase. The volume around the bead is called the **void volume**, V_o. It should be apparent the volume inside the bead is given by

$$V_i = V_t - V_o \tag{3.3.1}$$

A solute elutes from the column in a broad peak. If the sample volume applied to the column is very small compared to V_t, the volume at which a solute elutes, V_e, is considered the center of the elution peak. This is true when $V_{sample} \gg V_e$.

Suppose we view this chromatography as a solute partitioning between the mobile and stationary phases (the basis of all chromatography). In that case, we might be interested in what fraction of the stationary phase, V_i, a solute might partition into. Such a ratio would be given by:

$$K = \frac{V_e - V_o}{V_t - V_o} \tag{3.3.2}$$

V_t-V_o (= V_{inside}) represents 100% of the stationary phase, where K is a **distribution coefficient**. Consider two cases:

1. A very large solute compared to the pore size of the bead: In this case, V_e-V_o = 0 since V_e would be equal to V_o. (The solute wouldn't "see" any of the V_i.) In this case, K = 0. The solute would elute in the column's void volume since it is too large to partition into the volume within the beads. All solutes of molecular weight greater than or equal to the smallest solute that can't enter the gel beads will all elute in the void volume. Hence, solutes greater than this minimal size will co-elute from the column and not be separated. V_o is usually about 30-40% of the V_t.
2. A very small solute compared to the pore size. In this case, V_e-V_o = V_t - V_o, since V_e would be equal to V_t. The solute would "see" all of the solvent within the bead. In this case, K = 1. Like above, all solutes of MW equal to or less than the largest solute that can partition into the entire volume within a bead will co-elute at a volume near V_t.

Hence K is a **partition coefficient**, which varies from 0 - 1 and represents that fraction of V_i into which a solute could partition. However, this K is not exactly a partition coefficient since the actual volume of the gel matrix is assumed to be zero above. The graph in Figure 3.3.5 shows typical V_e as a fraction of V_t for solutes of different sizes (the x-axis is V_e/V_t).

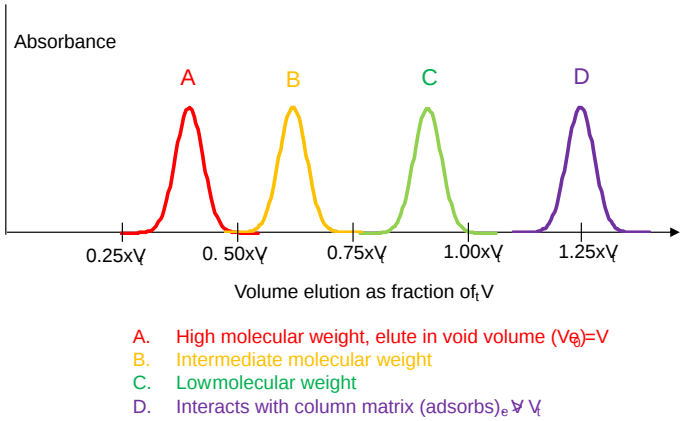


Figure 3.3.5: Elution volumes (V_e for different size macromolecules)

Large species that cannot enter the pores in the beads flow around it and elute in the void volume (V_0), which is about 35-40% of V_t (red bell-shaped curve). Very small species can partition into V_0 and V_i, so the elute near V_t (green bell-shaped curve). If a species adsorbs to the column bead through noncovalent interactions (such as hydrogen bonds or ion-ion interactions), it may elute after V_t (purple bell-shaped curve).

K depends on the size and shape of the solute. The size and shape of an object determine its flow properties in a fluid. Frictional resistance (itself a force that acts in the opposite direction to the velocity, another vector quantity) can be shown to be proportional to the velocity.

$$F_f \propto v \tag{3.3.3}$$

or

$$F_f = -fv \tag{3.3.4}$$

where f is the frictional coefficient, which depends on the shape. The bigger the object, the more frictional resistance to movement. For a sphere, it can be shown that:

$$f = 6\pi\eta R_s \tag{3.3.5}$$

where η is the viscosity (a measure of the resistance to flow of a liquid - water has a low viscosity, real maple syrup a high viscosity), and R_s (Stokes radius) is the radius of the hydrated sphere (the larger R_s, the larger the frictional coefficient, the larger the F_f which resists motion). For an irregularly shaped object, the Stokes radius is the radius of a sphere that would have the same frictional coefficient as the object. Hence, the R_s for a protein molecule that was not spherical would be much larger than the R_s for another protein molecule of identical molecular weight that was spherical. Hence, the V_e and the K values for a solute on a gel filtration column would best be related to the Stokes radius since R_s values consider both size and shape.

If you separate two proteins of equal mass but one is highly elongated, and the other is spherical, the elongated one, with a large R_S, would elute first (assuming that both don't elute together in the void volume, V_0.

Gel filtration can determine the molecular weight of an unknown, spherical (globular) protein compared to a standard curve generated from other globular proteins of known molecule weight. To ensure the protein has the same "effective" shape, the proteins are eluted under denaturing conditions to remove shape contributions to the elution order.

3.3.5.3: Separation based on charge - Ion Exchange Chromatography

The chromatography resin in this type consists of an agarose, acrylamide, or cellulose resin or bead, which is derivatized to contain covalently linked positively or negatively charged groups. Proteins in the mobile phase will bind through electrostatic interactions to the charged group on the column. In a mixture of proteins, positively charged proteins will bind to a resin containing negatively charged groups, like the carboxymethyl group, CM ($-OCH_2COO^-$) or sulfopropyl, SP, ($-OCH_2CH_2CH_2SO_3^-$). In contrast, the negatively charged proteins will pass through the column. The positively charged proteins can be eluted from the column with a mobile phase containing either a gradient of increasing salt concentration or a single higher salt concentration (isocratic elution).

The most positively charged protein will be eluted last, at the highest salt concentration. Likewise, negatively charged proteins will bind to a resin containing positively charged groups, like the diethylaminoethyl group, DEAE ($-OCH_2CH_2NH(C_2H_5)_2^+$) or a quaternary ethyl amino group, QAE, and can be separated analogously.

Ion exchange chromatography separates compounds according to the nature and degree of their ionic charge. The column used is selected according to its type and charge strength. Anion exchange resins have a positive charge and are used to retain and separate negatively charged compounds (anions). In contrast, cation exchange resins have a negative charge and are used to separate positively charged molecules (cations).

Before the separation begins, a buffer is pumped through the column to equilibrate the opposing charged ions. Upon sample injection, solute molecules will exchange with the buffer ions as each competes for the binding sites on the resin. The length of retention for each solute depends upon the strength of its charge. The most weakly charged compounds will elute first, followed by those with successively stronger charges. Because of the nature of the separating mechanism, pH, buffer type, buffer concentration, and temperature all play important roles in controlling the separation.

Figure 3.3.6 shows a ***cation exchange column***. The beads (brown) contain negatively charged functional groups that can bind positive protein (blue) or concentrated regions of positive charge on a protein.

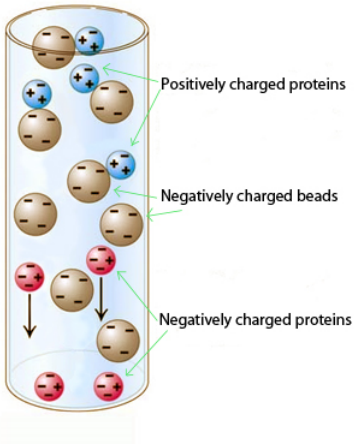


Figure 3.3.6: Ion Exchange Chromatography https://upload.wikimedia.org/wikiped...n_exchange.jpg. Jspiteri at English Wikibooks, Public domain, via Wikimedia Commons

Before loading the column with protein, the negatively charged beads would interact with positively charged countercations (often Na^+) from the column equilibration buffer. When the protein solution is introduced to the column, the positively charged protein will exchange with the bound Na^+ ions (hence the name cation exchanger). Conversely, an anion exchanger consists of positively charged beads, which exchange anions. Proteins bound through ion-ion interactions can be eluted by increasing the Na^+ concentration in the eluting solution stepwise or using a salt concentration gradient. Ion exchange chromatography is a powerful protein purification tool and is frequently used in analytical and preparative separations.

3.3.5.4: Affinity Chromatography

In this technique, the chromatography resin is derivatized with a group that binds to a specific site on a given protein of interest. It may be a group that binds to the active site of an enzyme (such as benzamidine-agarose, which is used to purify trypsin) or an antibody that recognizes a specific amino acid sequence (an epitope) on a protein. For example, an antibody can be made to a specific peptide from albumin, the antibody covalently linked to agarose, and the antibody-agarose column then used to purify albumin specifically. This is a powerful technique since antibodies can be made that will bind selectively to a single protein. Knowing only the DNA sequence of a protein that has never been previously isolated, the amino acid sequence of the unknown protein can be derived from the DNA sequence. A 10-12 amino acid peptide from that protein can be synthesized in the lab (see the last section below), and an antibody raised against the peptide. The antibody will most likely bind to the unknown protein and the peptide and could be used to purify the protein.

These features of affinity chromatography are illustrated in Figure 3.3.7.

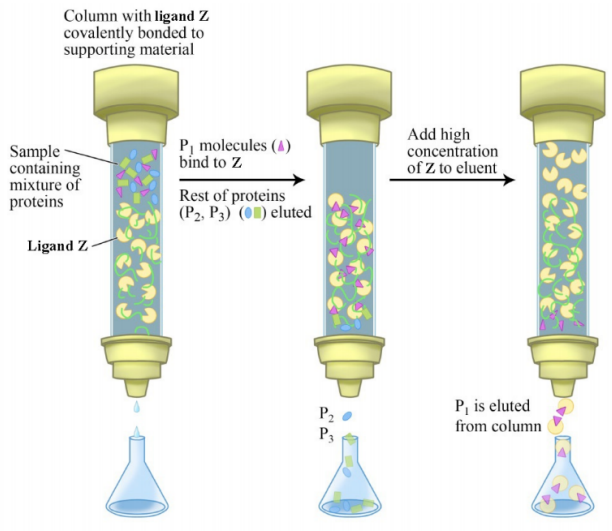


Figure 3.3.7: **Example of Affinity Chromatography.**

In this example in Figure 3.3.7, protein P1 has an affinity for ligand Z and will bind to the column, while proteins P2 and P3 will pass through the column. Protein P1 can then be eluted from the column using high concentrations of free ligand Z.

? In vitro peptide synthesis for antibody production

For more details on the chemistry of in vitro peptide synthesis, click the link below.

Answer

When making anti-peptide antibodies that recognize target proteins or studying an isolated peptide by itself, it is more difficult to isolate and purify a peptide from its original protein than to synthesize it in the lab using solid-phase synthesis. We describe this technique below.

Peptides are chemically synthesized by the condensation reaction of the carboxyl group of one amino acid to the amino group of another. Two chemical challenges must be addressed. The formation of an amide bond between the carboxylic acid of one amino acid and the amine of the other is thermodynamically unfavorable, so the carboxyl end must be activated typically by the reaction of the incoming amino acid with a reagent such as dicyclocarbodiimide. Secondly, reactive functional groups on the side chains and the amine of the carboxyl group-activated amino acid must be protected from unwanted reactions. Chemical peptide synthesis most commonly starts at the carboxyl end of the peptide (C-terminus) and proceeds toward the amino-terminus (N-terminus). Protein biosynthesis in living organisms occurs in the opposite direction. Chemical synthesis facilitates the production of peptides that incorporate unnatural amino acids, peptide/protein backbone modification, and the synthesis of D-amino acids.

The established method for producing synthetic peptides in the lab is known as solid-phase peptide synthesis (SPPS). Pioneered by Robert Bruce Merrifield, SPPS allows the rapid assembly of a peptide chain through successive reactions of amino acid derivatives on an insoluble porous support. The solid support consists of small, polymeric resin beads functionalized with reactive groups (such as amine or hydroxyl groups) that link to the nascent peptide chain. Since the peptide remains covalently attached to the support throughout the synthesis, excess reagents and side products can be removed by washing and filtration. This approach circumvents the comparatively time-consuming isolation of the product peptide from the solution after each reaction step, which would be required when using conventional solution-phase synthesis.

Each amino acid to be coupled to the peptide chain N-terminus must be protected on its N-terminus and side chain using appropriate protecting groups such as t-Boc (t-butyloxycarbonyl-, acid-labile) or fluorenylmethyloxycarbonyl (Fmoc, base-labile), depending on the side chain and the protection strategy used (see below).

The general SPPS procedure involves repeated cycles of alternate N-terminal deprotections and coupling reactions. The resin can be washed between each step to remove side products. Figure 3.3.8 shows the mechanism for the solid phase synthesis of a dipeptide.

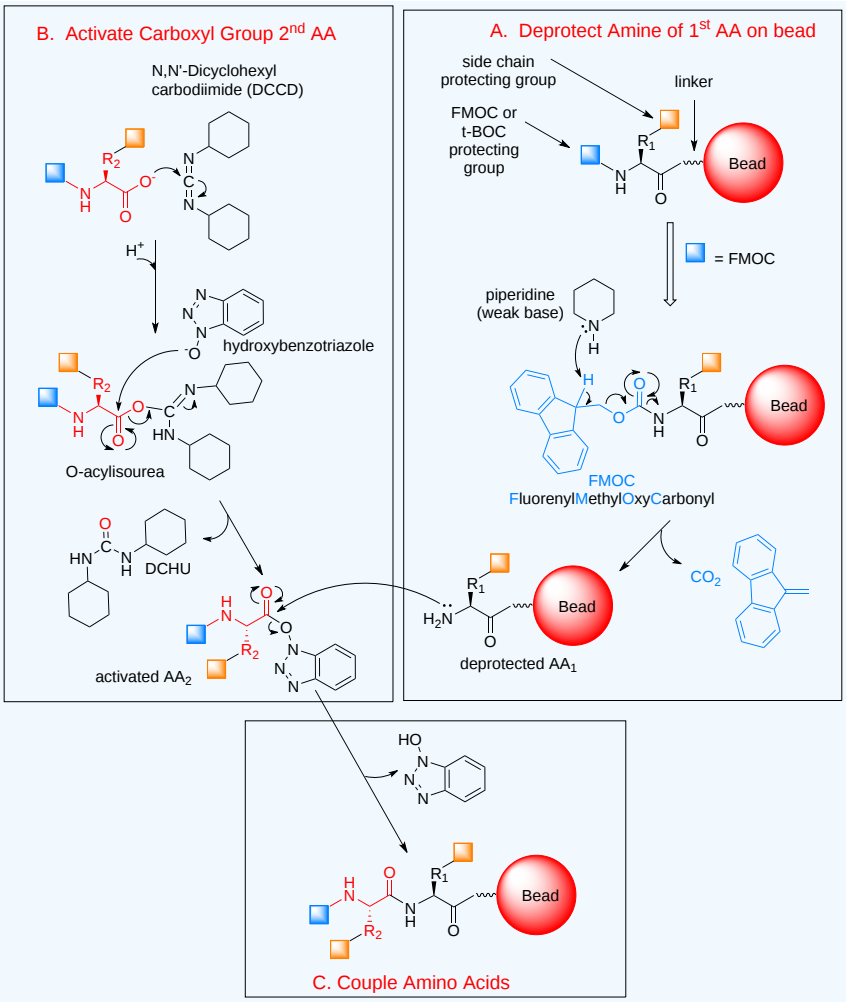


Figure 3.3.8: Solid phase peptide/protein synthesis

A. Deprotection of AA_1: The first amino acid is coupled to the resin or purchased pre-coupled. The amine terminus contacting an FMOC group is deprotected with piperidine. The hydrogen abstracted from the FMOC is acidic as its negatively charged conjugated base is aromatic since the negative charge on that C becomes sp^2 hybridized to create the aromatic anion. The weak base piperidine is used to avoid side reactions.

B. Activation of AA_2: The carboxyl group of AA_2 reacts with a carbodiimide, which is attacked by the carboxylate of AA_2, leading to the formation of an isourea derivative. This can react with a second nucleophilic catalyst (which is regenerated in step C), hydrobenzotriazole (HBT), to form the activated HBT ester and the very stable urea derivative.

C. Coupling Reaction: The activated AA_2 now reacts with the amine of solid phase N-terminal deprotected AA1 to form the peptide bond.

This cycle repeats until the desired sequence has been synthesized. At the end of the synthesis, the crude peptide is cleaved from the solid support while simultaneously removing all protecting groups using a reagent strong acid like trifluoroacetic acid. The crude peptide can be precipitated from a non-polar solvent like diethyl ether to remove organic soluble by-products and then purified using reversed-phase HPLC. The purification process, especially of longer peptides, can be challenging because small amounts of several byproducts, which are very similar to the product, must be removed. For this reason, so-called continuous chromatography processes such as MCSGP are increasingly being used in commercial settings to maximize the yield without sacrificing purity levels.

Reaction yields limit SPPS, and typically, peptides and proteins with 70 amino acids or more push the limits of synthetic accessibility. Synthetic difficulty is also sequence-dependent; aggregation-prone sequences, such as amyloids, are typically difficult to make. Longer lengths can be accessed by using ligation approaches, such as native chemical ligation, where two shorter, fully deprotected synthetic peptides can be joined together in solution.

Cell proteins can be increasingly engineered by manipulating their gene to contain a molecular tag, either a small peptide or a protein for which antibodies are commercially available. The tag is expressed at either the target's N- or C-terminal end to not interfere with the folding of the expressed target protein. Examples of peptide tags include the His (sequence HHHHHH), FLAG (sequence DYKDDDDK), and HA (YPYDVPDYA) tags. The HA tags derive from the influenza hemagglutinin protein. A small protein, such as the green Fluorescent Protein - GFP), can also be used as a tag. The resulting fusion protein of GFP connected to the target protein can also allow the target protein to be localized and followed by confocal fluorescence microscopy within the cell. Chromatography resins with covalently attached antibodies to the His, FLAG, HA peptide tags, and GFP are commercially available as affinity chromatography resins as shown in the right-hand side of Figure 3.3.9: below.

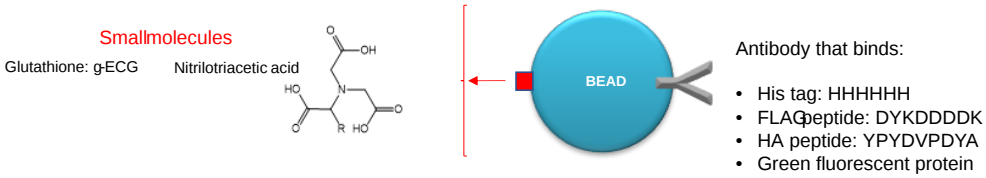


Figure 3.3.9: Protein Tags and small molecule bead ligands for affinity chromatography

Affinity reagents other than antibodies can be attached to the beads, as shown in the left-hand side of Figure 3.3.9. Two, in particular, are Ni-Nitrilotriacetic acid (Ni-NTA) and the short peptide glutathione (γ-gluatmylcysteinylglycine). They also bind tagged proteins. The Ni-Nitrilotriacetic binds the His tag by chelating the nickel ion with the 6 histidine imidazole groups on the His-tagged protein. (Note that His tags can also be bound to anti-His tag antibody beads.) Glutathione binds a protein tag, Glutathione-S-Transferase (GST), linked in a fusion protein to the target.

The structure of the Ni^{2+}-NTA complex attached to a bead and imidazoles (on a His_6 tag) are shown in Figure 3.3.10 below.

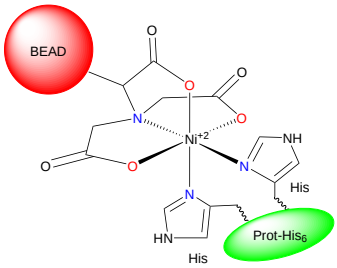


Figure 3.3.10: Ni^{2+}-NTA - imidazole complexes (after Wegner and Spatz, https://doi.org/10.1002/anie.201210317)

Figure 3.3.11 shows an interactive iCn3D model of the Ni^{2+} NTA complex.

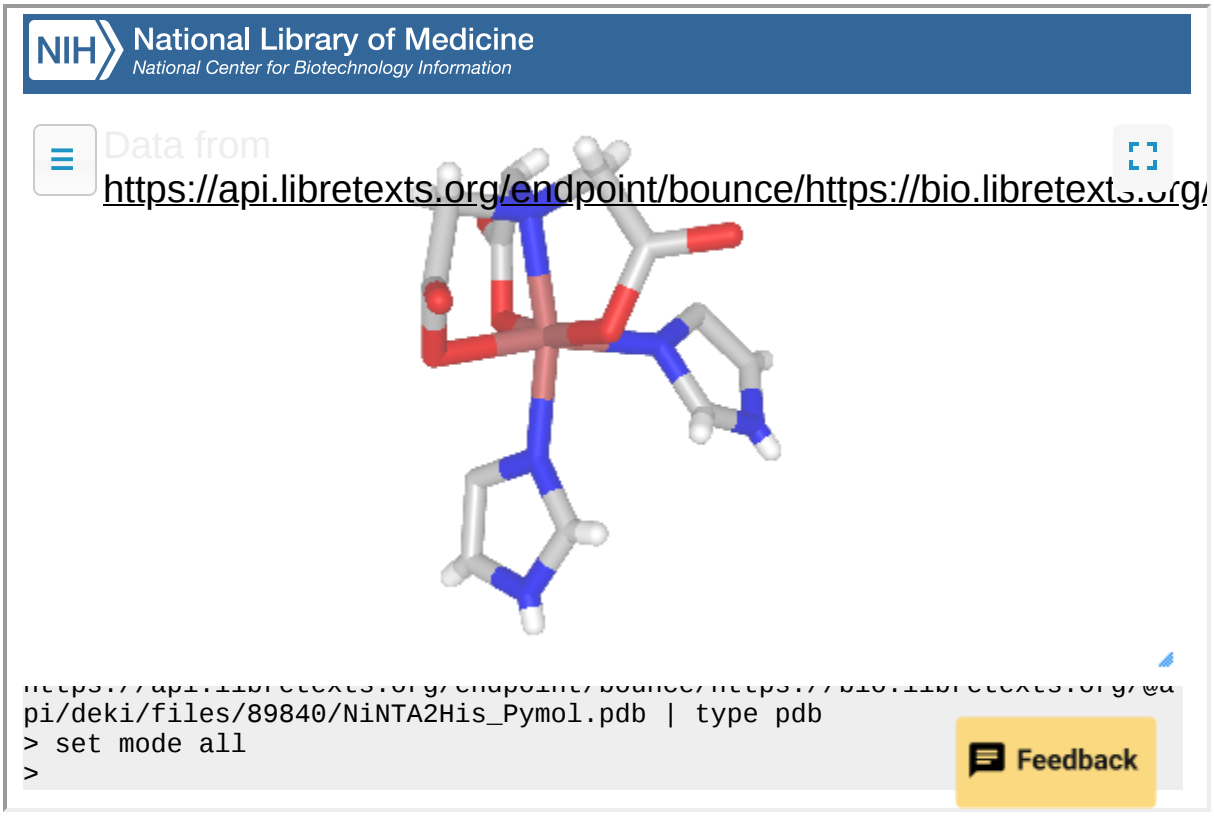


Figure 3.3.11: iCn3D model of the Ni^{2+}-NTA complex. To see the Ni^{2+} in spacefill, follow these commands in the iCn3D window. Click the ☰ menu icon and then:

- **Select, Select on 3D**, choose Atom
- Alt-click the central Ni ion
- **Style, Chemical, Sphere**

Without a His_6 tag, two water molecules occupy adjacent axial and equatorial positions (shown interacting with imidazole rings of His in the images above) in the generally octahedral complex. One ring C in the iCn3D models shows a carbon with an unfilled valence. The bead is covalently attached to that carbon.

The His tag, probably the most widely used, binds strongly to divalent metal ions such as nickel and cobalt. The protein can be passed through a column containing Ni-nitrilotriacetic. All untagged proteins pass through the column. The protein can be eluted with imidazole, which competes with the imidazole side chain on the His tag for binding to the column, or by a decrease in pH (typically to 4.5), which decreases the affinity of the tag for the resin. While this procedure is generally used to purify recombinant proteins with an engineered affinity tag (such as a 6xHis tag), it can also be used for natural proteins with an inherent affinity for divalent cations.

3.3.5.5: Hydrophobic Interaction Chromatography (HIC)

HIC media is similar to reverse phase chromatography in which a matrix like silica (very polar with exposed OH groups) is derivatized with ester or ether links from the silica surface hydroxyl OHs to nonpolar molecules, usually containing 8 or 18 carbons in the acyl or alkyl chain. Proteins with exposed hydrophobic groups would preferentially bind to the bead. The interactions of the protein with the derivatized beads are increased by adding high concentrations of salt to the aqueous solution, making water effectively more polar. This would shift the equilibrium towards binding the surface-exposed nonpolar region on the protein to the nonpolar C8 or C18 chains. The ionic strength of the buffer is then reduced to elute proteins in order of increasing hydrophobicity, as shown in Figure 3.3.11.

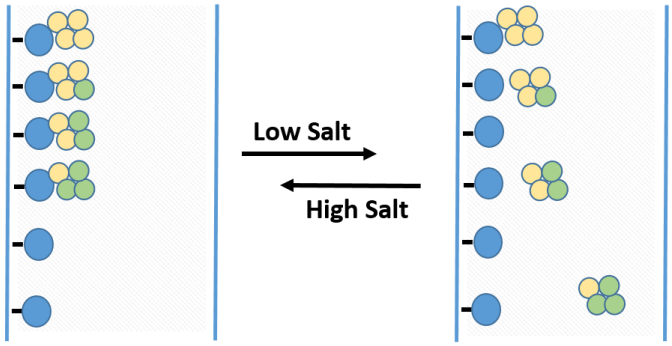


Figure 3.3.11: Hydrophobic Interaction Chromatography.

The column matrix, shown in blue, has a hydrophobic ligand covalently attached. In high salt conditions, proteins will bind to the matrix with differing affinity, with more hydrophobic proteins (shown in yellow) binding more tightly than more hydrophilic proteins (shown in green) When the salt concentration is decreased, more hydrophilic proteins will be released first, followed more hydrophobic proteins.

3.3.5.6: High Performance Liquid Chromatography (HPLC) and Fast Protein Liquid Chromatography (FPLC)

High-performance liquid chromatography or high-pressure liquid chromatography (HPLC) applies high pressure to drive the solutes through the column faster than the gravity-forced flow of solvent. The small and close-packed packing beads allow greatly increased resolution. Because of the close packing of the small beads, no flow would occur with an external pump. The most common form of HPLC is "reversed phase" HPLC, where the column packing material is hydrophobic. The proteins are eluted by a gradient of water and increasing amounts of an organic solvent, such as acetonitrile. The proteins elute according to their hydrophobicity. After purification by HPLC, the protein is in a solution that only contains volatile compounds and can easily be ***lyophilized (freeze-dried)***. HPLC purification frequently results in the denaturation of the purified proteins and is thus not applicable to proteins that do not spontaneously refold.

Due to the drawbacks of HPLC, an alternative technique using lower pressure was developed called ***Fast protein liquid chromatography (FPLC).*** In FPLC, the mobile phase is an aqueous solution, or "buffer". A positive displacement pump controls the buffer flow rate and is normally kept constant. In contrast, the composition of the buffer can be varied by drawing fluids in different proportions from two or more external reservoirs. The stationary phase is a resin composed of beads, usually of cross-linked agarose, packed into a cylindrical glass or plastic column. Depending on the application, FPLC resins are available in a wide range of bead sizes and surface ligands.

An ion exchange resin is typically chosen in the most common FPLC purification systems, as shown in Figure 3.3.12

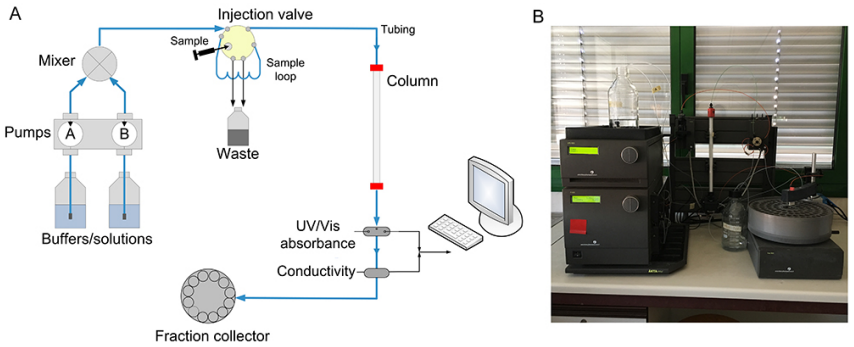


Figure 3.3.12: **Typical FPLC System.** A. Scheme of basic components and typical flow path for a chromatography system. B. Picrue of GE Healthcare AKTA FPLC apparatus. *Image provided by LaVerde, V., Dominici, P. and Astegno, A. (2017)* Bio-protocol *7(8): e2230.*

A mixture containing one or more proteins of interest is dissolved in 100% buffer A and pumped into the column. The proteins of interest bind to the resin while other components are carried out in the buffer. The total flow rate of the buffer is kept constant; however, the proportion of Buffer B (the "elution" buffer) is gradually increased from 0% to 100% according to a programmed change in concentration (the "gradient"). Buffer B contains high concentrations of the exchanger ion. Thus, as Buffer B's concentration gradually increases, bound proteins will dissociate depending on their ionic interactions with the column matrix and appear in the eluant. The eluant passes through two detectors, which measure salt concentration (by conductivity) and protein

concentration (by absorption of ultraviolet light at a wavelength of 280 nm). As each protein is eluted, it appears in the eluant as a "peak" in protein concentration and can be collected for further use.

3.3.6: Purification Scheme

During the protein purification process, a quantitative system is necessary to determine the total amount and concentration of total and target protein at each step, the biological activity of the target protein, and its overall purity. This will help guide and optimize the purification method being developed. Ineffective separation techniques can be disregarded, and other techniques that give higher yields and retain the protein's biological activity can be adopted.

Thus, each step in the purification scheme is quantitatively evaluated for the following parameters: total protein, total activity, specific activity, yield, and purification level. The terms are described below.

Pretend you are a researcher who wants to isolate a novel, unknown protein from a bacterial culture. You grow 500 ml of the bacteria overnight at 37ºC and harvest the bacteria by centrifugation. You remove the culture broth and retain the bacterial pellet. You then lyse the bacteria using freeze/thaw in 10 mL of reaction buffer. You then centrifuge the lysed bacteria to remove the insoluble materials and retain the supernatant containing the soluble proteins. Your protein of interest has a biological activity you can measure using a simple assay that causes a color change in the reaction mixture, as illustrated in Figure 3.3.13. You also note that this reaction rate increases with increasing concentrations of your protein supernatant.

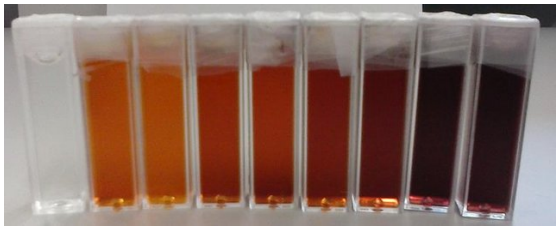


Figure 3.3.13: **Example of a Chemical Reaction that causes a color change from orange to brown depending on increasing concentration.** *Image from: Ludwig, N., et. al. (2015) on* Research Gate

At this point, you can measure your baseline concentrations for the first purification level (bacterial lysis and removal of insoluble proteins and other cellular debris by centrifugation).

Total Protein is calculated by measuring the concentration in a fraction of the sample and then multiplying that by the total volume of your sample. In this case, you are starting with 10 mL of supernatant. In a typical assay to measure protein concentration, you will use 50 - 200 µL of sample to determine the protein concentration. For example, if you calculate that there is 7.5 µg/µL in your initial assay, you would need to convert that value into mg/mL and then multiply it by 10 mL for a total of 75 mg of protein in 10 mL of supernatant (Table 3.3.1).

Total Activity is measured as the enzyme activity within the assay multiplied by the total volume of the sample. For example, you might use 5 to 50 µL of the sample in your biological reaction. If you calculated the activity in your assay to be 2.5 units/µL, this would be equivalent to 2,500 units/mL or 25,000 units/10 mL of supernatant. Note that the ***enzyme unit***, or international unit for the enzyme (symbol U, sometimes also IU), describes the ***enzyme's catalytic activity***. 1 U (µmol/min) is defined as the amount of the enzyme that catalyzes the conversion of one micromole of substrate per minute under the specified conditions of the assay method.

Specific Activity is measured by dividing the Total Activity by the Total Protein. In our example, 25,000 units divided by 75 mg of protein = 333.3 units/mg.

Yield measures the biological activity retained in the sample after each purification step. The amount in the first step is set to be 100%. All subsequent yield steps will be evaluated using the first purification step. It is calculated by dividing the current step's total activity by the first step's total activity and multiplying it by 100.

The ***Purification level*** evaluates the purity of the protein of interest by dividing the specific activity calculated after each purification step by the specific activity of the first purification step. Thus, the first step always has a value of 1. A typical purification analysis scheme is shown below in Table 3.3.1.

Purification Step	Purification Method	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Yield (%)	Purification Level
1	Cell Lysis - Centrifugation	75	25,000	333.3	100	1
2	Salting Out	54	23,416	433.6	93.7	1.3
3	Ion Exchange Chromatography	12.3	21,227	1725.8	84.9	5.2
4	Size Exclusion Chromatography	0.93	18,633	20,035	74.5	60.1

Table 3.3.1: A typical purification analysis scheme.

Note that after each purification step, the ***total protein*** goes down, as you separate the target protein from other proteins in the mixture. ***Total Activity*** also goes down with each purification step, as some of your protein of interest is also lost at each purification step because (1) some protein will stick to the test tubes and glassware, (2) some protein won't bind with 100% efficiency to your column matrix, (3) some protein may bind too tightly to be removed from the column matrix during elution, and (4) some protein may be denatured or degraded during the purification process.

The overall ***percent yield*** **(or recovery)** for each purification step represents the amount of your protein of interest that is retained. Alternative purification methods should be explored if the percent yield is too low.

Note that in a good protein purification scheme, the ***specific activity*** should go up substantially with each level of purification, as the amount of your protein of interest makes up a greater percentage of the total protein within that fraction. If the specific activity only increases modestly within a purification step, or if it decreases during a purification step, this could indicate that (1) your protein of interest is being substantially lost at that step, (2) your protein of interest is being denatured or degraded and is no longer biologically active, or (3) that a required cofactor or binding protein is being reduced at that purification step. Additional experiments may need to be conducted to determine which causes predominate so that steps can be taken to reduce protein inactivation. For example, many proteins are temperature sensitive and will degrade or denature at room temperature. Completing purification steps on ice can often reduce degradation.

The **fold-increase** (specific activity at step n/specific activity initial) or ***purification level*** should increase during purification. A high-quality purification procedure results in a high fold increase or purification level AND a high recovery or yield.

3.3.7: Electrophoresis: Separation and Analysis

In column chromatography, flow through the column is driven by hydrostatic pressure, causing flow from higher regions of higher pressure at the top of the column reservoir to lower pressure (drops eluting from the bottom of the column). Ultimately, the hydrostatic pressure (in columns not driven by mechanical pumps) derives from the gravitational force. However, proteins are also charged particles and can be moved by an external electric field instead of a gravitational field. Electrophoresis is the movement of charged particles in an electric field. As we will show below, the movement of a charged protein within a static matrix in the presence of an external electric field depends on both size and shape. Electrophoresis can be used for both analytical and preparative separations of proteins. The most common uses are for analytical separations.

3.3.7.1: Theory

What determines how a protein moves in an electric field? Consider a charged particle (+Q) moving in an electric field (E) in a nonconducting medium, such as water. Suppose the particle is moving at a constant velocity toward the cathode (- electrode where cations go). In that case, the net force F_{tot} on the particle is 0 (since F=ma, and the acceleration (a) of the particle is zero at constant velocity). Two forces are exerted on the particle, one F_E, the force exerted on the charged particle by the field, which is in the direction of the motion (toward the cathode), and the other, F_f, the frictional force on the charged particle, which retards its motion toward the cathode, and hence is in the direction opposite to the motion (toward the anode (+) electrode). This is shown in the Figure 3.3.14:

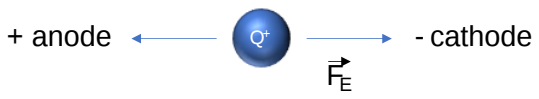


Figure 3.3.14: Movement of charge Q^+ in an electric field

Therefore:

$$F_{tot} = F_E + F_f \tag{3.3.6}$$

where F_E, the electrical force, is

$$F_E = QE \tag{3.3.7}$$

and

F_f, the frictional force, is

$$F_f = -fv \tag{3.3.8}$$

In the last equation, v is the particle's velocity, and f is a constant called the frictional coefficient. This equation shows that the force F_f hindering motion toward the cathode is proportional to the particle's velocity. This is intuitive since one would expect the higher the velocity, the greater the $F_{f,}$ which would hinder the motion. The frictional coefficient depends on the size and shape of the molecule. The larger the molecule, the larger the frictional coefficient (i.e. more resistance to the molecule's motion). It can be shown that the frictional coefficient for a spherical particle is given by

$$f = 6\pi\eta R_S \tag{3.3.9}$$

where η is the viscosity (a measure of the resistance to flow of a liquid - water has a low viscosity, real maple syrup a high viscosity), and R_s (Stokes radius) is the radius of the hydrated sphere (the larger R_s, the larger the frictional coefficient, the larger the F_f which resists motion toward the cathode). This equation should be intuitive based on your experiences.

When the velocity of the charged particles is constant (i.e, there is no net force on the particle), $F_e = F_f$, and using equations 3.3.6-8 gives

$$QE = fv \tag{3.3.10}$$

Hence v/E = Q/f = U = the **electrophoretic mobility**, or

$$U = \frac{V}{E} = \frac{Q}{6\pi\eta R_S} \tag{3.3.11}$$

Therefore, the electrophoretic mobility U is proportional to the **particle's charge density (charge/size, Q/Rs)**, not just the size, as is the case for spherical proteins in size exclusion chromatography. Macromolecules of different **charge densities** can thus be separated by electrophoresis. This discussion deals with the simplest case since, in reality, there are counter ions in the solution (from salts), which would form a cloud around the charged macromolecule and partially shield the charged particle from the electric field E.

Modern-day electrophoresis is conducted in solid gels (such as polyacrylamide) formed from liquid acrylamide solutions after adding a polymerizing agent. The solid gel is porous to solute and solvent molecules. It serves as a medium for electrophoresis while helping to eliminate convection forces in the liquid that interfere with the separation. Electrophoretic experiments have been conducted on the space station in weightless conditions to prevent such perturbations.

One complication that affects this idealized description of electrophoresis in polyacrylamide gels is that the gels have pores through which the macromolecules move. Think of the protein moving under an electric force through a "spider web-like" matrix. As in gel chromatography, the smaller molecules can pass through the pores more readily than larger molecules, so an additional sieving

mechanism contributes to the *effective* mobility. (Also, the gel could alter the local effective electric field). The sieving effect of the gel increases the resolving power of this technique.

It has been determined that the actual electrophoretic mobility of the protein, U, is a function of the mobility of the protein in a concentrated sucrose solution (Uo) and T, the total concentration of the acrylamide in the polymerized gel. The higher the concentration of acrylamide in the unpolymerized gel solution, the smaller the size of the pores in the polymerized gel. An equation showing the relationship between U, Uo, and T is shown below:

$$\log U = \log U_0 - K_r T \tag{3.3.12}$$

where K_r is the slope of a log U vs T plot for a given protein. Since K_r is a function of the radius of the molecule, it is possible to determine the molecular weight of a protein molecule by performing several electrophoretic separations in gels of different acrylamide concentrations (T) and extrapolating results to T = 0, hence eliminating pore size effects. Problems arise, however, if the proteins are **not** spheroid in shape.

Is there any way to obtain molecular weight information, in addition to purity determination, on a single gel? What would result if two different proteins with the same molecular weight and total net charge but different shapes were run on a single acrylamide gel? The more elongated shape (large Stokes radius) would have lower electrophoretic mobility ($U = Q/6\pi\eta R_s$). A larger Rs would also cause the protein to enter the pores more slowly. Hence, electrophoretic mobility and sieving effects would cause this protein to run anomalously slowly and have a higher apparent molecular weight. Also, imagine two globular proteins of different sizes but with compensatory charge differences, which might allow the two proteins to migrate at the same speed in the gel.

An astute reader might quickly recognize a problem separating proteins by gel electrophoresis. Some proteins are negatively charged (pH > pI), some would be neutral (pH=pI), and the rest would be positive (pH < pI). Only some proteins would enter the gel and move to the electrode at the bottom of the gel. Luckily, there is a way to eliminate both charge and shape effects in the electrophoresis of proteins, and that is to run the gel under denaturing conditions when all proteins have the same charge density. The denaturant of choice for electrophoresis is usually sodium dodecyl sulfate (SDS), which is an ionic detergent with the structure $CH_3(CH_2)_{10}CH_2OSO_3^-$ (a single chain amphiphile). This detergent binds to and denatures most proteins, with about 1.4 g SDS binding/g of protein (about 1 SDS/2 amino acids). Since there is 1 negative charge/SDS, the binding of SDS masks any of the charges on the protein and gives all proteins an overall large negative charge. *Additionally*, SDS-proteins complexes have been shown to have an elongated cylindrical-like shape generally. Since the amount of SDS bound per unit protein mass is constant, the overall charge density on all proteins is similar, so the electrophoretic mobility is only determined by sieving effects.

SDS also eliminates shape differences in the proteins as a variable since all proteins have the same general rod-like shape. (The use of SDS is analogous to using 8M urea in the gel chromatographic separation of proteins to determine molecular weights). Mobility becomes only a function of the molecular weight of the protein and not its shape. The molecular weight of an unknown protein can be determined by comparing the protein's position on an SDS polyacrylamide gel with a series of known molecular weight standards from which a linear plot of the ln Mr vs R_f can be used to calculate unknown molecular weights. This is similar to the analysis in gel chromatography, where ln Mr is a linear function of K_{avg}, the distribution coefficient when the gel is run under denaturing conditions. However, some proteins run anomalously on such gels (due to incomplete or excess binding SDS), so alternative techniques of molecular weight determination should be used in conjunction with this technique.

Proteins are usually heated in SDS to 100 ^oC for 3 minutes, in the presence of a reducing agent such as β-mercaptoethanol (βME), to denature the protein to a rod-shaped protein. Apparent molecular weight can be obtained under non-reducing conditions (without βME), but these should be considered just estimates. Running proteins, both in the presence and absence of the reducing agent, can provide important information on the subunit structure of a protein. A multimeric protein whose subunits are held together by disulfide bonds can be resolved into component subunits by adding the reducing agent. If the subunits are held together only by noncovalent interactions, they will separate under denaturing conditions (SDS), eliminating subunit interactions in the presence or absence of β-ME.

3.3.7.2: Electrolytic vs Galvanic Cells

Electrode nomenclature can be confusing. In the above example, in the presence of an existing electric field produced by a power source, the +Q particle, a cation, moved to the cathode, the negative electrode. In protein or DNA electrophoresis, you know that the negatively charged proteins or DNA fragments, which are anions, move to the bottom of the gel towards the anode, the positive electrode. However, when you studied galvanic cells (that spontaneously produce current without a power supply) in general

chemistry courses, the cathode is the positive electrode, and the anode is the negative electrode. Instead of remembering the charge state of the electrode, it is better to focus on the redox reactions that occur at each electrode:

- It is always true that Oxidation occurs at the Anode - both start with a vowel - and Reduction occurs at the Cathode - both start with a consonant!

In galvanic cells, an electrical current is generated from a spontaneous set of redox half-reactions. In electrolytic cells, an external power supply is required to drive the motion of the particle and the nonspontaneous redox reactions that occur at the electrodes. In electrophoretic cells for protein analysis, the bubbles released at the electrodes are from the electrolysis of water - $2H_2O(l) \rightarrow 2H_2(g) + O_2(g)$. The oxidation number of H goes from +1 to 0 in the reaction so H is reduced and is produced at the cathode. In SDS-PAGE, SDS-coated proteins (negatively charged) migrate to the anode, the positive electrode in this case. Commercial electrolytic cells can produce $Cl_2(g)$ and Mg(s) from the aqueous electrolyte $MgCl_2(aq)$. Figure 3.3.15 illustrates the differences between galvanic and electrolytic cells.

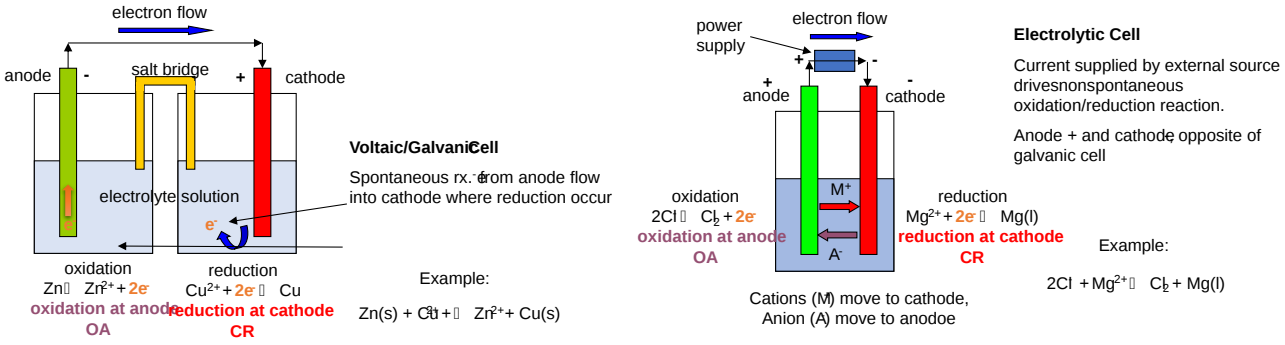


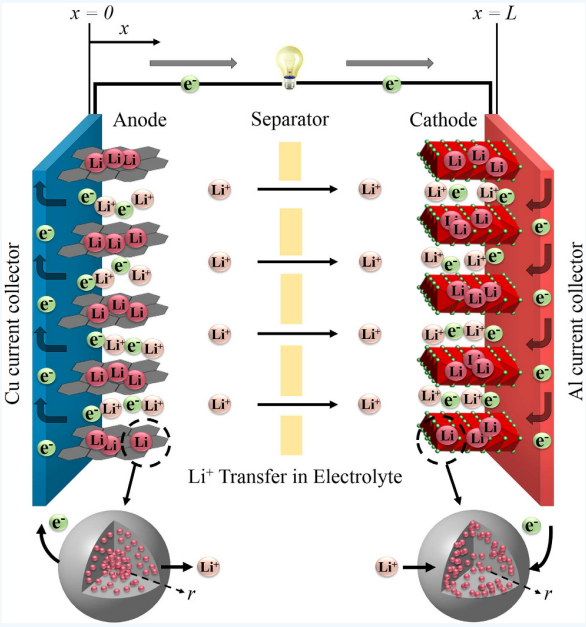
Figure 3.3.15: Galvanic vs electrolytic cells

? Lithium Ion Batteries

Optional: To support the material in Chapter 32: Biochemistry and Climate Change, to review your learning in previous courses, to prepare for more complex redox reactions (mitochondrial electron transport, photosynthesis, and nitrogen fixation) that we will encounter later in this book, and as we move to electric vehicles (EVs) to reduce greenhouse gases from burning fossil fuels, it's important to know how Li^+ ion batteries that power EVs work. All you need is basic general chemistry, whose ideas we need to understand biochemical redox reactions.

Click the link below if you wish to learn more about lithium ion batteries

The diagram below shows the components of a Li^+ ion battery that discharges to power electronic devices (in the case of a light bulb) and electric cars. Compare it to the spontaneously discharging galvanic/voltaic cell (left image above).



Schematic of the involved species/charge transports and electrochemical reactions in lithium-ion cells during discharge. Lithium atoms are shown as red spheres, Li^+ ions as light orange, and electrons as light green. Jiang, F., Peng, P. Elucidating the Performance Limitations of Lithium-ion Batteries due to Species and Charge Transport through Five Characteristic Parameters. *Sci Rep* **6**, 32639 (2016). https://doi.org/10.1038/srep32639. Creative Commons Attribution 4.0 International License. http://creativecommons.org/licenses/by/4.0/

In a lithium-ion battery:

* The - electrode (anode, where oxidation occurs) is porous carbon graphite (parallel sheets of graphene) with Li atoms/ions on a conducting metal (Cu). This is where Li^+ ions and free electrons (e^-) are formed. The electrons move through the electric circuit to the + electrode (the cathode);

* A liquid electrolyte solution (replaces the "salt bridge" in the Cu/Zn^{2+} cell) allows the transfer of Li^+ to the + cathode (the tricky part to remember) to preserve charge neutrality.

* The + electrode (cathode, where reduction occurs) is a metal oxide (such as CoO_2) on a conducting metal (Al), which reduces the arriving Li^+ ions to neutral L and intercalation into the CoO_2 polymer. This is the reaction shown in the figure above. Most references state that the Co(IV) in CoO_2 is reduced to Co(III), which is perhaps more likely given the low ionization energy of the gaseous form of Group 1 Li.

Here are the actual chemical reactions that occur at the anode and cathode on the discharge of the battery to power a light or an electric car:

Li atoms (neutral, red spheres) in the graphite anode are oxidized to Li^+ ions (yellow spheres) as shown in this reaction:

$$\text{Oxidation at anode: } LiC_6 \rightarrow C_6 + Li^+ + e^-$$

The Li^+ ions move from the anode through the electrolyte to the cathode side, where they get reduced (like Cu^{2+} in the left figure above). Here is the reaction:

$$\text{Reduction (at cathode): } Li^+ + e^- + CoO_2 \rightarrow LiCoO_2$$

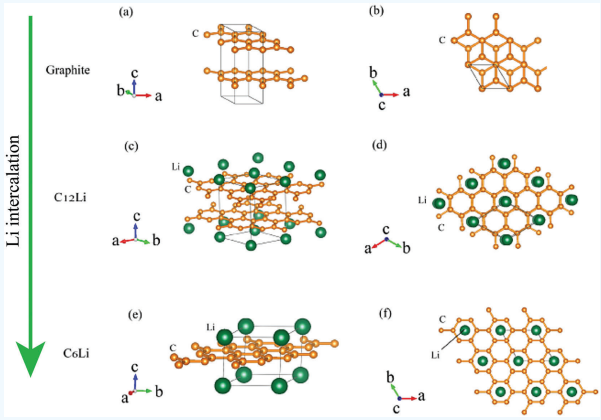
The last reaction could indicate that either Li^+ or Co(IV) is reduced to maintain charge balance. The oxidation number of Co in $CoCO_2$ is +4 (as each O is -2). In $LiCoO_2$, if Co is reduced, it becomes Co +3 as it gains an electron, with the +1 on Li balancing the net -4 on the two oxygens. If Li^+ is reduced, it becomes atomic Li^0. This presumes that the interactions are all ionic. So with charge states/oxidation numbers, this could be written as:

$$Li^+ + e^- + Co(IV)(O^{2-})_2 \rightarrow [Li^+Co(III)(O^{2-})_2 \text{ or } Li^0Co(IV)(O^{2-})_2]$$

A better way might be to envision a sharing of the electron between the Li^+ and Co(IV) ions. We'll address this more below.

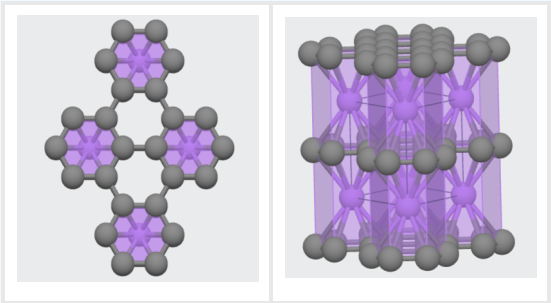
On charging (nonspontaneous, driven by plugging in the car), the reverse process occurs as reduced Li in $LiCoO_2$ gives up an electron (as Zn in the left figure above) to form $Li^{+,}$ which moves to the graphite electrode. Here is an animation (which sometimes works on refreshing the browser) showing the structure and Li-ion diffusion out of a LiCoO2 cathode (click the Lithium Ion Diffusion Icon).

The figure below shows the 3D structures of different graphite forms with intercalated Li in the anode.



The crystal structure of (a) and (b) graphite, (c) and (d) $C_{12}Li$, and (e) and (f) C_6Li. (b, d, and f) Show a c-axis view of graphite, $C_{12}Li$, and C_6Li, in which Li is located at the center of six-member carbon rings. ***Phys. Chem. Chem. Phys.***, 2017,**19**, 19058-19066. Creative Commons Attribution-NonCommercial 3.0 Unported

Here are two views of the LiC_6 anode structure (see this link for a 3D view). Carbons in graphite are shown as gray spheres, while Li is shown as purple spheres. Lithium migrates in a 2D plane between 2 graphene layers (graphene is a single layer of graphite). Here is another link for the structure of LiC_6.



The empirical formula for this structure is LiC_6, but it is also written as C_6Li. If we consider LiC_6 as purely an ionic compound, the lithium would appear to have a +1 charge and oxidation state, and each of the Cs would be -1/6 = -0.166, with the electron from Li entering the pi cloud of graphite. But didn't we say above that Li likely has a 0 oxidation number/charge (i.e., it's a neutral atom) in the LiC_6 structure?

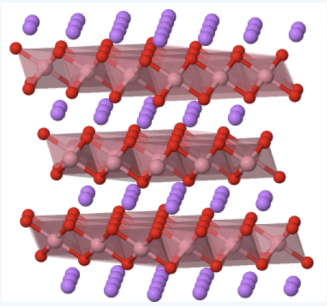
Here is some help for this problem. Assigning electrons for charge state in metal ion complexes is arbitrary. It doesn't indicate the actual electron density around an atom (such as in the case of H_3O^+, in which O has a formal charge of +1 but has almost all of the electron density). In the case of a transition metal complex, we can consider the bond between the metal and ligand to be a coordinate covalent bond. For a transition metal complex, the metal's oxidation number can be determined by first separating (in your mind) the ligand and its donating electrons from the metal. There are 2 ways to do this:

* send both electrons in the coordinate covalent bond to the withdrawn ligand (ie. a heterolytic cleavage). If the ligand is $-NH_3$, the removed ligand has a full lone pair ($:NH_3$) and is neutral. For a $-CH_3$ ligand, the removed ligand would be $:CH_3-$, a carbanion with a negative charge.

* send one electron to the withdrawn ligand and one to the metal (i.e a homolytic cleavage). If the ligand is -CH_3, the removed ligand is $\cdot CH_3$ (free radical and neutral).

For the case of Li, a nontransition Group I metal, if we assign one electron in the bond to Li and the other to the separated C_6 electron cloud, the oxidation state of Li would be 0, consistent with the anode reaction shown above. Given the delocalized electron density from the pi clouds of the graphite interacting with Li, a coordinate covalent bond is a better descriptor than a simple ionic one. At the same time, given how easily Li gives up an electron, it makes sense to show both Li and Li^+ in the electrodes.

Here is a figure showing the $LiCoO_2$ cathode.



Lithium is shown in purple, oxygen in red, and Co in orange. https://www.chemtube3d.com/lib_lco-2/

Much research is devoted to finding new electrons and metal substitutes to make them cheaper and more environmentally favorable. Lithium manganese oxide ($LiMn_2O_4$) and lithium iron phosphate ($LiFePO_4$) are alternatives for the cathode, which don't rely on expensive and toxic cobalt.

3.3.8: Polyacrylamide Gel Electrophoresis - PAGE

Electrophoresis is performed in a porous yet solid medium to eliminate any problems associated with convection currents. Such media are formed from solidifying a liquid solution of agarose (used mostly for electrophoresis of DNA fragments and very large proteins) or the polymerization of a solution of acrylamide. Polymerization of acrylamide is initiated by the additions of ammonium persulfate in the presence of tetramethylenediamine (TEMED), along with a dimer of acrylamide (N,N'-methylene-bis(acrylamide) connected covalently between the amide nitrogens of the acrylamides by a methylene group. The structures of these compounds are shown in Figure 3.3.16.

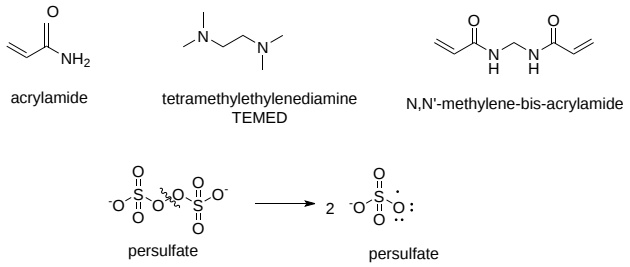


Figure 3.3.16: Structures of reagents use to make PAGE gels

As shown above, the acrylamide's free radical polymerization is initiated by adding ammonium persulfate, which forms free radicals on dissolving in water.

The radical initiates polymerization of the acrylamide, as shown below. The TEMED, through its ability to exist as a free radical, acts as an additional catalyst for polymerization. A rigid gel is only formed, however, when N,N'-methylene-bis(acrylamide) is added to the mixture during the polymerization, which cross-links adjacent acrylamide polymers as shown in Figure 3.3.17.

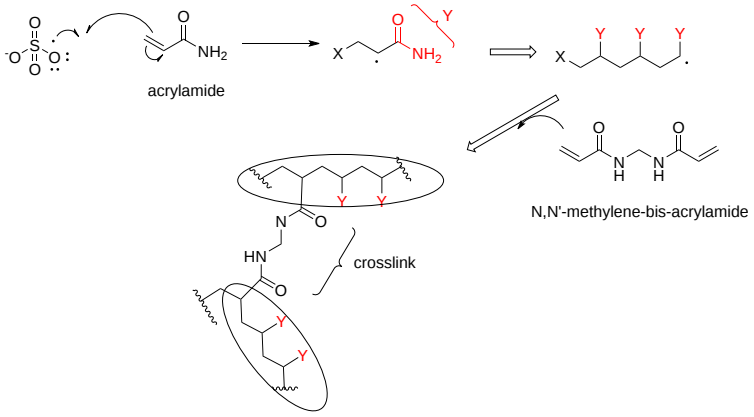


Figure 3.3.17: Polymerization of acrylamide

The amount of bisacrylamide added during polymerization controls the degree of cross-linking and, hence, the pore size of the polymerized gel. The effect of pore size is **OPPOSITE** to that in gel chromatography. In both cases, large proteins have a difficult time entering the pore. In gel chromatography, large proteins partition preferentially into the mobile liquid phase (the void volume) and are eluted most **QUICKLY** from the column. In electrophoresis, large proteins, which can not readily enter the pores in the gel, are not as easily transported by the electric field through the gel and elute most **SLOWLY**. Pore size can not be controlled as accurately as in the manufacture of gel chromatography resins.

How do proteins migrate through the gel? A viscous protein solution is layered on the top of the gel in a small well molded into the gel during the polymerization process. The bottom and top parts of the gel are inserted into reservoirs containing a buffered solution and the appropriate electrode. The electric field is applied, and the proteins migrate through the hydrated gel. The nature of the buffer solution in the reservoir and the polymerized gel is important. The buffer components must not bind to the proteins to be separated. Additionally, for native (non-denatured gels), the pH of the medium must be such that the proteins have the appropriate charge so they will migrate in the expected direction.

There are many variations of electrophoresis commonly used. Gels can be polymerized in tubes or slabs and the presence or absence of denaturing agents. Additionally, a given slab might consist of two separate slabs polymerized on top of the other, each with a different acrylamide concentration and pH value. The top part is the stacking gel, the bottom is called the running gel. Other gels have a continuous gradient of acrylamide concentrations (from low at the top to high at the bottom). Most commercially available precast gels use continuous acrylamide concentration gradients. Figure 3.3.18 shows a gel placed in an electrophoresis chamber.

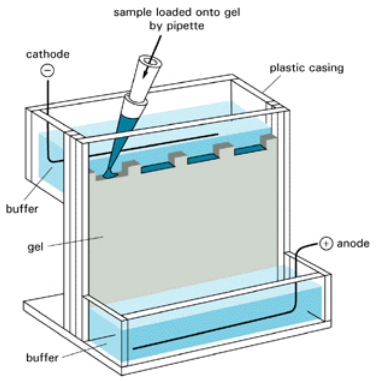


Figure 3.3.18: Electrophoresis gel and chamber https://openwetware.org/mediawiki/in...&oldid=1096467

Whether the gel has a continuous gradient or is discontinuous, the top part of the gel is a low concentration acrylamide (2-4%), often in a Tris HCl buffer solution (pH 6.5), usually 2 pH units below that used in the running gel. The lower part of the gel is 8-15% acrylamide, depending on the choice of gel, which is selected based on the molecular weight of the proteins to be separated. The upper buffer reservoir contains Tris-buffered with a weak acid such as glycine (pKa2 = 9.6) to the same pH as the running gel.

Proteins electrophorese quickly through the low-concentration stacking gel at the top of continuous gradient gels and effectively "stack" as they hit the interface between the stacking and running gels or before they enter too far into the continuous gradient gel.

This increases the compactness of the proteins before they enter the "running" section of the gel and increases resolution.

For discontinuous gels, how does this stacking process work? When the electrophoresis is started, glycine ions from the upper reservoir (at pH 8.7) enter the stacking gel since, at that pH, they have an average partial negative charge. The stacking gel buffer ions continue moving in the stacking gel, but when the glycine ions enter the pH 6.5 of the stacking gel, they become zwitterions with a zero net charge and hence stop their motion toward the anode. The electrical resistance in the stacking gel increases since the number of ions moving through the stacking gel decreases. To maintain a constant current throughout the circuit, there will be a *localized* increase in the voltage *in the stacking gel* (from Ohms Law, V=iR). This will cause the proteins to migrate quickly and all stack in a single, very thin disc right behind the Cl- ions in the stacking gel (which are in front because they have the highest charge density and electrophoretic mobility of any ion in the stacking gel). The proteins will not pass the Cl- ions since, if they did, they would immediately slow down since they would no longer be in an area of diminished charged carriers and higher voltage. At the stacking gel/running gel interface, the proteins can not all migrate at the same speed due to the sieving effects of the more concentrated gel. They will be separated in the running gel. The glycine eventually enters the running gel, assumes its fully charged state at that pH (8.7), passes the proteins, and restores the deficiency in charge that occurred in the stacking gel.

3.3.8.1: Detection of proteins in the gel:

Most proteins do not absorb at visible wavelengths of light and hence will not be visible during electrophoresis. To ensure that the proteins are not eluted from the gel into the lower buffer reservoir, a small molecular weight, anionic dye, and bromophenol blue are added to the protein before it is placed on the gel. The electrophoresis is halted when the dye reaches the bottom of the gel. The gel assembly is removed from the electrophoresis chamber, the glass plates separated, and the gel washed into a series of solutions to render the banded proteins visible.

- Coomassie Brilliant Blue dye is the most common stain in labs. It is dissolved in a methanol/acetic acid solution, generating significant waste. Proteins bind this dye, with a concomitant spectral shift in the absorbance properties of the bound dye. The methanol and acetic acid in the dye solution also help to "fix" the proteins in the gel and prevent their diffusion into the solution. After the gel is stained, the background stain is removed with acetic acid/methanol, leaving the blue-colored protein bands. Some proteins will not be stained with Coomassie blue. A "colloidal Coomassie Blue" stain doesn't use methanol or acetic acid, so it is commonly used. A simple water solution can destain the gel.
- Silver staining: This involves the reduction of Ag(I) to elemental silver and its deposition by protein in the appropriate reaction solutions, much as in a photographic process. (Remember, in the BCA assay, peptide bonds reduce Cu(II) to Cu(I), which is chelated to BCA.) A developer and fixer solution is required. This technique is 10-50 X more sensitive than Coomassie Blue staining. Figure 3.3.19shows gels stained with Coomassie Blue (A)and silver staining (B).

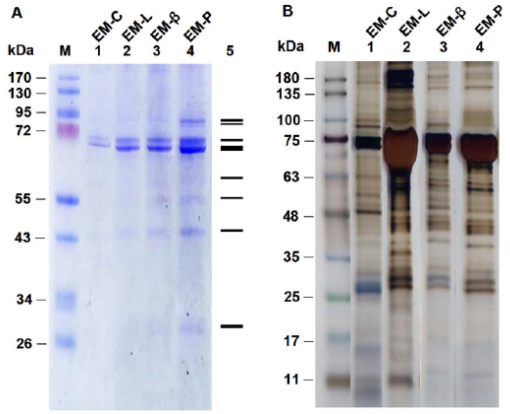


Figure 3.3.19: PAGE gels stained with Coomassie Blue (A) and silver stain (B) Chen Y-Y, Chen J-C, Lin Y-C, Kitikiew S, Li H-F, Bai J-C, et al. (2014) Endogenous Molecules Induced by a Pathogen-Associated Molecular Pattern (PAMP) Elicit Innate Immunity in Shrimp. PLoS ONE 9(12): e115232. https://doi.org/10.1371/journal.pone.0115232. Creative Commons Attribution License

- Modifying proteins with fluorescent or radioactive tags before electrophoresis: This offers greater sensitivity. After the electrophoresis of a radiolabeled protein, the gel can be dried and overlaid with X-ray film for periods as long as months, if necessary, to allow sufficient film exposure by a low-concentration protein. This visualization technique is called autoradiography.

3.3.8.2: Variations on polyacrylamide gel electrophoresis:

Isoelectric focusing: In this technique, a pH gradient is set up within the polyacrylamide gel or strip. This is accomplished by pre-electrophoresing a series of low molecular weight molecules containing amino and carboxyl groups called **ampholytes,** each with a different isoelectric point. When subjected to an electric field, the most negative of the species will concentrate at the anode, while the most positive will concentrate near the cathode. The remaining ampholytes will migrate in between as ampholytes migrate to their isoelectric point and set up a linear pH gradient in the gel.

Proteins initially in regions with a pH below its isoelectric point are positively charged and migrate toward the cathode. In contrast, those in a media with pH lower than its pI will be negatively charged and migrate towards the anode as shown below in Figure 3.3.20. The migration will lead to a region where the pH coincides with its pI. There, the protein will have a zero net charge and stop. Thus, amphoteric molecules are located in narrow bands where the pI coincides with the pH. In this technique, the point of application is not critical, as molecules will always move to their pI region. The stable pH gradient between the electrodes is achieved using a mixture of low molecular weight ampholytes whose pIs cover a preset pH range.

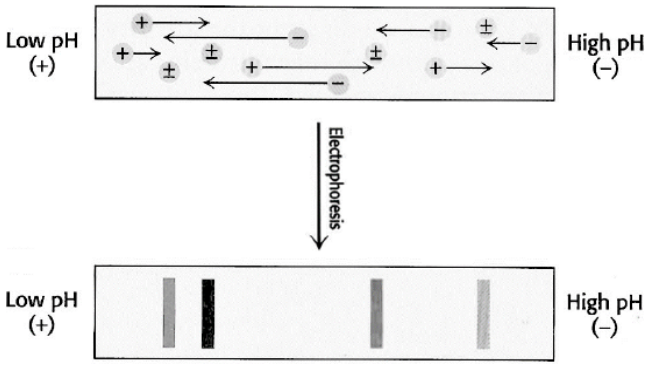


Figure 3.3.20: Isoelectric Focusing. A pH gradient is established in a gel before loading the sample. After the sample is loaded a voltage is applied. The protein will migrate to its isoelectric pH, at which they have no net charge. *Image from Magdeldin, S.*

2D electrophoresis: Two-dimensional gel electrophoresis (2-DE) is based on separating a mixture of proteins according to two molecular properties, one in each dimension. The most used is based on a first dimension separation by isoelectric focusing (IEF) and a second dimension according to molecular weight by SDS-PAGE. A conditioning step is applied to proteins separated by IEF before the second-dimension run. This process reduces disulfide bonds and alkylates the resultant sulfhydryl groups of the cysteine residues. Concurrently, proteins are coated with SDS for separation based on molecular weight. After the IEF, the tube or strip is placed across the top of a slab gel and subjected to SDS-polyacrylamide gel electrophoresis in a direction 90^{o} from the initial isoelectric focusing experiment. Figure 3.3.21 shows a stained 2D electrophoresis gel.

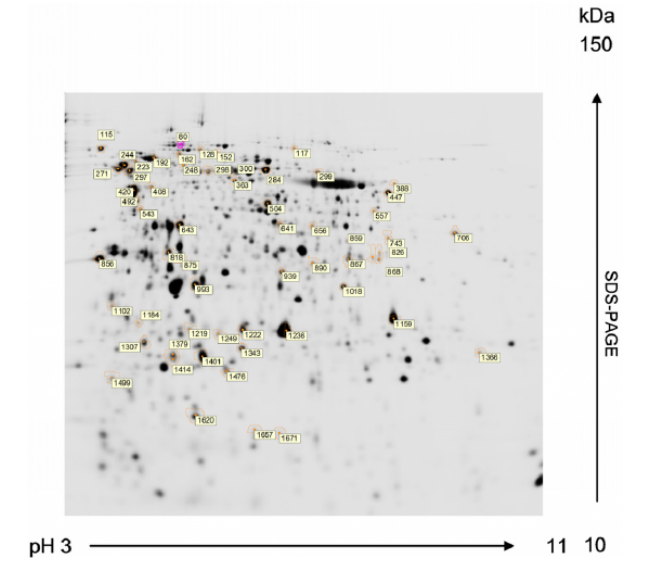


Figure 3.3.21: **Two-Dimentional Gel Electrophoresis.** *Image from Magdeldin, S*

In Figure 3.3.21, proteins of *Chlamydomonas reinhardtii are* resolved by 2-DE from preparative gels stained with MALDI-MS compatible silver reagent for peptide mass fingerprinting analysis. First dimension: isoelectric focusing in a 3-11 pH gradient. Second dimension: SDS-PAGE in a 12% acrylamide (2.6% crosslinking) gel (1.0 mm thick). Numbered spots marked with a circle correspond to proteins compared to be subsequently identified by MALDI-TOF MS. The MALDI-TOF MS analysis of protein sequences is discussed in more detail in Chapter 3.4.

One of the biggest problems in 2-DE is the analysis and comparison of complex mixtures of proteins. Currently, there are databases capable of comparing two-dimensional gel patterns. These systems allow automatic comparison of spots to identify those needed in the quantitative analysis precisely. Once interesting proteins are identified, they can be excised from gels, destained, and digested to prepare for mass spectrometry. This technique is known as peptide mass fingerprinting. The ability to precisely determine molecular weight by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and to search databases for peptide mass matches has made high-throughput protein identification possible. Proteins not identified by MALDI-TOF can be identified by sequence tagging or de novo sequencing using the Q-TOF electrospray LC-MS-MS.

Western blotting: After a standard SDS-slab electrophoresis experiment, the gel is overlaid with a piece of nitrocellulose membrane. The sandwich of gel and filter paper is placed back into an electrophoresis chamber such that the proteins migrate from the gel into the nitrocellulose, where they irreversibly bind. This is illustrated in the figure below. Note, however, that in the absence of staining, the protein bands in either the PAGE gel or Western blot would not be visible. Standards (lane 5) would be visible if they were labeled with chromophores, as shown in Figure 3.3.22

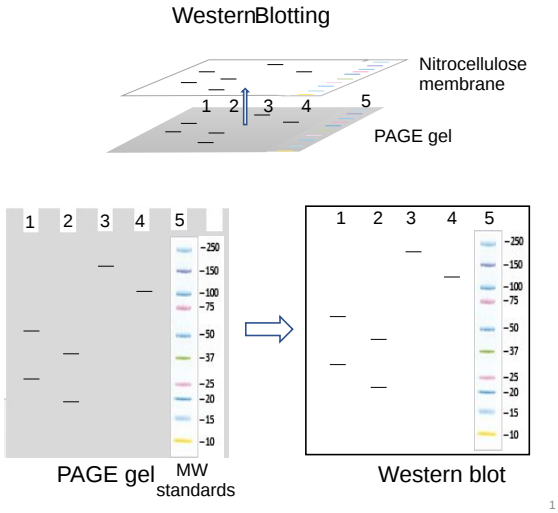


Figure 3.3.22: Western blotting of proteins

If a cell lysate were applied to a lane of a PAGE gel, the bands would appear as overlapping smears on the stained gel after staining with any technique. Western blots are useful because specific bands can be visualized (stained) on the nitrocellulose membrane using a detection system linked to an antibody that recognizes a specific target protein. This is illustrated in Figure 3.3.23

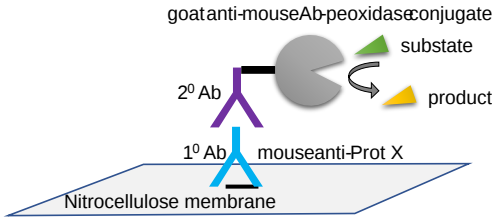


Figure 3.3.23: Developing a Western blot

3D electrophoresis: To detect specific proteins in a 2D electrophoresis experiment, a 3rd dimension of separation, a Western blot, could be performed on the PAGE gel and the nitrocellulose stained with an antibody specific to a target protein. That is illustrated in Figure 3.3.24

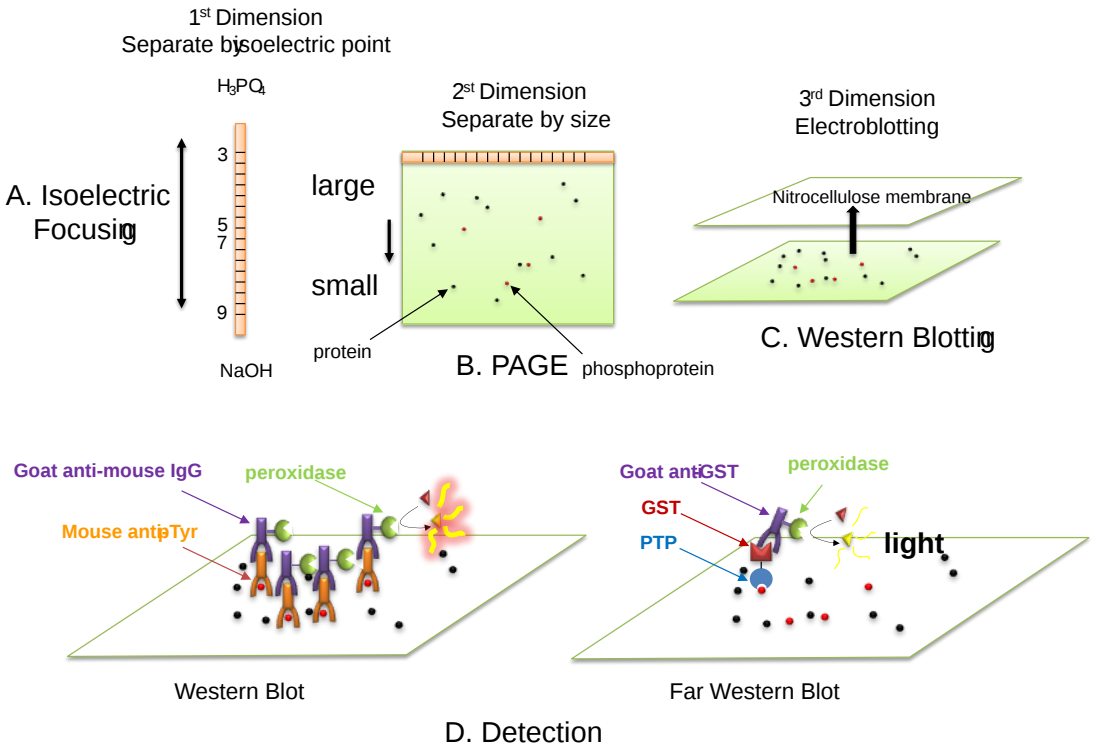


Figure 3.3.24: "3D" electrophoresis - Isoelectric focusing, PAGE and Western blotting

Part A, isoelectric focusing, is followed by a PAGE gel (B). The red dots represent proteins that have undergone a post-translational modification in which a phosphate group has been added to tyrosine side chains (for example). Western blotting is performed in panel C, and staining is performed in panel D. The left blot in D uses an antibody that recognizes phosphorylated tyrosine side chains on protein. The right blot is D is sometimes called a Far Western blot. If the protein on the nitrocellulose membrane retains some 3D native structure or can be induced to refold, it can be probed on the blot by a protein that binds to the native form of the protein on the blot. In the example shown in panel D above, the p-Tyr-protein target on the nitrocellulose membrane recognizes a fusion protein of PTP-GST. GST is a protein tag for detection. PTP is a protein tyrosine phosphatase, an enzyme that hydrolyzes p-Tyr on specific phosphorylated target proteins.

3.3.9: Summary

This chapter provides a comprehensive overview of protein purification and electrophoretic analysis, two essential methodologies in biochemistry for isolating and characterizing proteins. The chapter begins by emphasizing the necessity of protein purification, given the vast diversity of proteins in cells and tissues. It describes how inherent differences—such as size, charge, hydrophobicity, and binding affinity—are exploited to isolate a single protein from complex biological mixtures.

Sample Processing and Initial Fractionation:
The process starts with sample processing where cells or tissues are disrupted using methods such as freeze/thaw cycles, sonication, or detergent treatment. Centrifugation is then used to separate soluble proteins from cellular debris. Special attention is given to preventing proteolysis by performing procedures at low temperatures and adding protease inhibitors.

Bulk Purification Techniques:
Precipitation using ammonium sulfate is introduced as an economical initial step to concentrate proteins while maintaining their native state, followed by dialysis to remove excess salt. The chapter then details various chromatographic techniques:

- **Size Exclusion Chromatography (Gel Filtration):** Separates proteins based on size and shape by exploiting differences in how they access the internal pore volume of the resin beads.

- **Ion Exchange Chromatography:** Separates proteins based on their net charge; proteins bind to charged resins and are eluted by altering the ionic strength or pH of the buffer.
- **Affinity Chromatography:** Uses specific ligand-receptor interactions (e.g., His-tag binding to Ni-NTA or antibody-antigen recognition) to achieve high selectivity in isolating the protein of interest.
- **Hydrophobic Interaction Chromatography (HIC):** Exploits differences in protein hydrophobicity by modulating salt concentrations to promote binding to nonpolar ligands on the resin. Advanced methods such as HPLC and FPLC are also discussed as ways to increase resolution and throughput in protein separation.

Quantitative Evaluation of Purification:
The chapter stresses the importance of tracking purification efficiency through quantitative metrics like total protein, total activity, specific activity, yield, and fold-purification. These measurements are crucial for optimizing purification protocols and ensuring the integrity of the target protein.

Electrophoretic Analysis:
The latter portion of the chapter focuses on electrophoresis, a technique that separates proteins based on their size, charge, and shape under the influence of an electric field. Both native and denaturing methods are covered:

- **SDS-PAGE:** By binding to proteins and imparting a uniform negative charge, SDS standardizes the electrophoretic mobility of proteins so that separation is primarily based on molecular weight.
- **Isoelectric Focusing (IEF):** Separates proteins based on their isoelectric points (pI) by establishing a pH gradient.
- **Two-Dimensional Electrophoresis (2-DE):** Combines IEF and SDS-PAGE to achieve high-resolution separation of complex protein mixtures. Detection methods, including Coomassie Blue and silver staining, as well as Western blotting and autoradiography for enhanced sensitivity and specificity, are also described.

This chapter integrates the theoretical and practical aspects of protein purification and analysis. It highlights the sequential nature of the purification process—from cell lysis to chromatography and electrophoretic separation—and underscores the importance of quantitative evaluation at each step to ensure high purity and activity of the isolated protein. This foundation is critical for subsequent structural, functional, and proteomic studies in biochemistry.

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